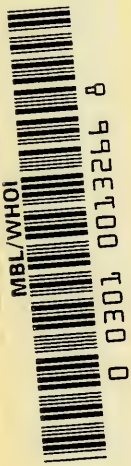
INFLUENCE OF TEMPERATURE ON BIOLOGICAL SYSTEMS

Frank H. Johnson, *Editor*



INFLUENCE OF TEMPERATURE ON BIOLOGICAL SYSTEMS

INFLUENCE OF TEMPERATURE ON BIOLOGICAL SYSTEMS

Incorporating papers presented at a symposium held at the University of Connecticut, Storrs, Connecticut, on August 27 and 28, 1956

Sponsored and published under the auspices of the Society of General Physiologists, with the support of the National Institutes of Health

Frank H. Johnson, *Editor*

Washington, D.C. 1957
AMERICAN PHYSIOLOGICAL SOCIETY

Library of Congress
Catalogue Card Number
57-14555

PRINTED IN THE UNITED STATES OF AMERICA
BY WAVERLY PRESS, INC., BALTIMORE, MD.

For his basic contributions to physiology, including his effective development and use of myothermic methods in elucidating the mechano-thermal relations and fundamental dynamics of muscular contraction, this symposium presented during the seventieth anniversary year of his birth, is dedicated to A. V. Hill, *with appreciation and respect.*

Contents

Introduction

The purpose of the symposium on which this book is based was to present, by means of a series of papers dealing with representative problems, a cross-section of contemporary research on temperature relations of biological processes at various levels of complexity, extending from the purely molecular, up through cells, tissues and organs, to whole organisms. In the light of present knowledge it was considered appropriate to include, as an aspect of the same subject, papers dealing primarily with the action of hydrostatic pressure. From several points of view, a symposium embodying both of these aspects may be considered a timely one.

The timeliness of the symposium, and the appropriateness of including papers on hydrostatic pressure, cannot be fully appreciated without reference to the general background as well as to the present status of research in this broad and fundamental area. More than passing comment, therefore, is called for concerning the manner in which studies on the biological effects of temperature and pressure began and progressed, as well as the advances that brought these seemingly unrelated fields of endeavor together, and the potential usefulness of such studies as avenues of approach to the understanding of basic mechanisms involved in the control of physiological processes.

With the advent of the Arrhenius theory of chemical reaction rates, in 1889,[1] a theoretical basis became available for interpreting biological rates in terms of laws applicable to ordinary chemical reactions. Various enzyme reactions in solution, as well as more complex processes in living cells, were soon found to conform in some measure to the Arrhenius equation; frequently, within certain ranges of temperature below the 'optimum' of a given process, the log of the rate turned out to be a linear function of the reciprocal of the absolute temperature. At temperatures only slightly above the optimum, the rate of thermal destruction in many instances also conformed to the Arrhenius equation. At intermediate temperatures, however, more complicated functions were encountered. Although these complications became the subject of much discussion and some interesting ideas, no convincing, quantitative theory to explain them emerged.

[1] References to the literature cited in this preface may be found in a recent book, *The Kinetic Basis of Molecular Biology*, by Johnson, Eyring and Polissar (Wiley, New York, 1954), which treats the biological effects of temperature and pressure from the point of view of modern reaction rate theory.

After a flourishing period of temperature studies that resulted, between two and four decades ago, in the accumulation of a fairly voluminous literature, interest began to wane. It seemed that this approach, while certainly not exhausted, held a diminishing promise of advances in any real understanding. Research with pressure had independently reached a similar impasse. It was not until around 1935 that certain new results in theory and experiment, as mentioned presently, removed some stumbling blocks in the way of further progress and laid a common basis in a rational chemical theory for the biological effects of both temperature and pressure.

At almost the same time that Arrhenius published his rate theory, Regnard published, in 1891, the first monograph dealing with the influence of increased hydrostatic pressures on biological processes. Interest in the physiological effects of pressure stemmed from the discovery, on the dredging expedition of the *Talisman* in 1882–1883, of living organisms at depths of 6,000 meters in the sea, where the pressure amounts to about 600 atmospheres or nearly 9,000 pounds per square inch. The early experiments, principally by Regnard and by his contemporary Certes, involved pressures only within the range from 1 to 600 atmospheres, and it is aproximately this range, i.e. up to about 1,000 atmospheres, that turns out to be most significant from a physiological point of view. Within this range, compression frequently results in large and easily reversible changes in activity.

Unfortunately, the studies begun by Regnard and Certes have been carried further by relatively few investigators, and the emphasis in research with pressure has tended in the direction of higher and higher pressures, upwards of 5,000 atmospheres, which cause irreversible, destructive effects such as the denaturation of proteins, inactivation of viruses and killing of bacteria. Moreover, very little work has yet been done with organisms indigenous to the deep sea, so the problem that inspired the first biological research with pressure remains virtually untouched except by implication of results that have been obtained with material from terrestrial sources. The implications of studies on terrestrial forms, however, can be remarkably enlightening, as several papers in this volume illustrate.

For nearly half a century after the appearance of Arrhenius's theory and Regnard's monograph, three stumbling blocks remained in the road toward understanding the physiological effects of pressure and temperature. First, there was no explicit theory for the quantitative relation between pressure and chemical reaction rates. Second, it was not realized that the physiological effects of increased pressure are subject to profound modification, even reversal, by temperature. Third, no clear evidence was

available that, under appropriate conditions, the thermal denaturation of proteins is quantitatively reversible on cooling; in consequence, there was no reason to believe that reversible protein denaturation played a key role in some of the important effects of temperature or, as we now know, of pressure as well. Thus it is understandable that Bělehrádek's comprehensive, detailed book on temperature and life processes, published in 1935,[2] contained no reference to pressure, and Cattell's thorough review of the physiological effects of pressure, published in 1936, contained scarcely more than a passing reference to temperature.

It was about this time, however, that the stumbling blocks referred to above were removed through coincidental advances whose significance in the over-all picture was not immediately realized. The contribution of most general importance was Eyring's theory of absolute reaction rates which, in 1935, became possible through advances in quantum physics and other fields bearing on chemical kinetics. This theory provided a rational basis for the interpretation of the relation between chemical reaction rates and pressure as well as temperature. In the same and preceding year, Brown presented data that clearly indicated a fundamental relationship between the influence of temperature and pressure on muscular contraction. In addition, his data indicated that a similar relationship among different organisms accustomed to different environmental temperatures was correlated with their specific ranges in temperature for physiological activity. Finally, by 1935, the research chiefly of Anson, Mirsky, Northrop and Kunitz on enzymes and other proteins established that thermal denaturations can be reversed, in part or in full according to the conditions of the experiment, and the suggestion had been made (Anson and Mirsky, 1931) that such reactions might be involved in controlling physiological activities. A few years later these independent advances were brought together and consolidated in studies of bacterial luminescence.

Experiments on bacterial luminescence by Brown, Johnson and Marsland in 1941–42 revealed some basic similarities in the effects of temperature and pressure on muscular contraction and on luminescence intensity, respectively. A new relationship was also observed, viz. between hydrostatic pressure and the action of narcotics; at the normal "optimum temperature" of luminescence, the inhibitory effect of alcohol, urethane or certain other agents disappeared under increased pressure. In collaboration with Eyring and his associates, a quantitative hypothesis was worked

[2] A recent book, *Temperatur und Leben*, by Precht, Christopherson and Hensel (Springer, Berlin, 1955) includes an extensive compilation of the literature on biological temperature relations since Bělehrádek's monograph, and includes some reference to pressure also.

out, on the basis of the theory of absolute reaction rates and ordinary equilibrium theory, which though admittedly oversimplified accounted for the data on luminescence with considerable success. The hypothesis included some new concepts, e.g. that the thermal denaturation of proteins is subject both to retardation in rate and to reversal by hydrostatic pressure (accounted for in terms of large, molecular volume increases of activation and of reaction, respectively); that the thermal denaturation equilibrium is involved in the physiological effects of certain narcotics, whose action consists, in part, in promoting the reversible and irreversible denaturation of a key enzyme system; that enzyme reactions may be accompanied by large volume changes of activation; and that the optimum temperature of a biological reaction occurs at a point where the effect of heat in accelerating enzyme activity is balanced by the effect of heat in promoting reversible enzyme denaturation. In terms of this hypothesis, several phenomena became understandable, e.g. the inhibitory action of pressure at low temperatures (relative to the specific optimum) where the denaturation equilibrium is normally not significant; the favorable action of pressure at high temperatures, where a large proportion of limiting enzyme exists in a catalytically inactive state that is reversible by pressure; the reversal of narcotic action by pressure; the reversible changes in the observed rate of the over-all process at normal pressure, i.e. the temperature-activity curve, over a broad range of temperatures from well below to considerably above the optimum temperature; and the changes in the net effects of pressure at a series of constant temperatures below, at and above the optimum.

Although the hypothesis arrived at through studies of bacterial luminescence was initially based on purely kinetic evidence pertaining to a process in intact, living cells, investigations of other systems and through other approaches have provided further evidence consistent with the original views. For example, the thermal denaturation of pure proteins in solution has been shown, both in this country and abroad, to be retarded by increased hydrostatic pressure. Perhaps the most remarkable example has to do with the relation between hydrostatic pressure and narcosis: tadpoles immersed in narcotizing concentrations of alcohol or urethane have been observed to recover their activity under increased pressure (Johnson *et al.*, 1950–51), and single nerve cells rendered inexcitable by these same drugs recover their excitability under increased pressure (Tasaki and Spyropoulos, this volume).

Other aspects of the research on luminescence, however, are more important than whether or not the original hypothesis has been justified in its various details by subsequent investigations. First of all, it brought

together the key advances in theory and observations referred to in the preceding paragraphs. Second, it demonstrated the usefulness of both temperature and pressure in basic biological research. And third, it emphasized the importance of relationships between temperature and other factors, such as hydrostatic pressure, in seeking to understand fully the influence of any one of these factors.

The usefulness of both temperature and pressure in theoretical biology, and the importance of inter-relationships between the effects of temperature, pressure and the chemical environment or state of biological systems are again emphasized by the papers in this volume, especially those on muscular contraction, cell division and nerve activity. Brown's paper on muscle, for example, elucidates certain aspects of muscular contraction in a measure that could scarcely have been achieved through any other approach, and at the same time provides a basis for understanding the specific differences in muscle physiology among organisms temporarily accustomed or genetically limited to different habitats. For, the same basic system, through relatively simple changes in the chemical environment, assumes different optimum temperatures, different optimum pressures and different over-all energies of activation.

Now, where do we stand at the moment, and what is the outlook for future research on the biological effects of temperature?

Recently, for the first time in approximately a generation, two books[1, 2] dealing extensively with biological temperature relations have been independently published. The present volume presents, in more detail than could be treated in either of the other two books, both new data and reviews on representative problems of general interest to biology. These three new books evidence a renewed and promising interest in the subject. Some questions have been answered, at least in part, while others seem well along the way toward solution, and still others are clearly more remote of solution. Divers new practical, as well as theoretical, problems have come to the fore, such as those pertaining to life in arctic and tropical environments, the action of fluctuating temperatures in preventing injury to both plants and insects, the influence of sudden temperature changes in synchronizing cell division, the relative temperature-insensitivity of 'biological clocks', etc. At the same time, new knowledge and improved means of investigating molecular structure are available, the kinetic complexities of rapid reactions and sequences of reaction are being resolved, and much more is known now then heretofore concerning the pathways and catalysts of intermediary metabolism. Thus it is reasonable to believe that we stand on the threshold of major new advances through research with temperature and the factors which modify its influence on biological systems.

The editor gratefully acknowledges the generous and able cooperation of all who participated in arranging as well as contributing to the symposium and to the publication of the results. The work of the individual authors, the support of the National Institutes of Health, the assistance of Dr. W. R. Amberson, Dr. C. Stacy French, Dr. W. D. McElroy and Dr. David F. Waugh as chairmen of the several symposium sessions, and the aid of Dr. Milton O. Lee and the publications office of the American Physiological Society, all deserve especial thanks.

F. H. Johnson
Princeton, N. J., 1957

THE CRITICAL COMPLEX THEORY OF BIOGENESIS[1]

HENRY EYRING AND FRANK H. JOHNSON, *University of Utah, Salt Lake City, Utah, and Princeton University, Princeton, New Jersey*

MUTATION followed by survival of the fittest accounts for most types of adaptation of organisms. However, the living world with its optical activity would possess precisely the same fitness for survival if it could all be reflected through a mirror. Such a reflection would transform all molecules into their mirror images and instead of the configurationally related *l*-amino acids as we now find them, we would have their *d*-amino counterparts. The resulting world would be exactly as efficient as the one we now have with no change in its chemistry, and hence no change in the relative fitness for survival of the various organisms. How optical activity arose in the living world in the first place is a perennially interesting question. It is interesting that in excellent discussions of biogenesis the significance of the universality of the *l*-amino acid is generally not stressed (1–5).

To understand the origin of optical activity in living things we require explanations within the framework of known physical and chemical principles of at least three facts: *a*) the fact that the molecular systems making up living things are optically active rather than racemic, as all uncatalyzed chemical preparations are; also *b*) that the different amino acids within an organism are configurationally related; and *c*) that the different amino acids from different species are likewise alike, i.e. all of the *l*-configuration. All of these points are answered if we assume that since all optically active molecules are made by the mediation of optically active templates, then these templates, which at present select configurationally related amino acids, must have originated in the remote past from a single optically active parent template. To explain how one template got the ascendancy over its optical isomer, we must assume that the first template to be successfully formed effectively excluded its rival from being formed and in addition flooded the world with replicas of itself. We now consider the whole matter from a somewhat broader perspective.

TIME AND CHANGE

It is usually supposed that around five billion years ago our universe assumed something like its present form. Associated with the emergence of

[1] Aided in part by a contract with the Office of Naval Research.

our solar system there was presumably intense nuclear evolution. Two or three billion years later when the intensity of nuclear radiation had abated sufficiently and temperatures had subsided to near present levels, all sorts of chemicals began accumulating. These compounds were synthesized by various influences. Among the contributing agencies was radiation from nuclear decay, as well as absorbed ultra violet radiation. Lightning was a third substantial contributor, and finally the carbides, which would be expected in a reducing world where oxygen had not yet been liberated by photosynthesis, would yield a multitude of compounds arising from polymerization and other reactions of acetylene. With neither free atmospheric oxygen nor biological enzymes to destroy the accumulating organic compounds, all sorts of complex molecules must have appeared in ever increasing concentrations and in widely scattered pools all over the land.

Such molecules would react and be elaborated into all types of complex systems, and since the amino acids would automatically appear in a world containing NH_3, CO_2 and H_2O along with radiation, electrical discharges and acetylene, we may suppose that eventually amino acids inevitably combined to form polypeptides, and eventually even simple proteins and rudimentary enzymes. Likewise phosphoric acids, the pentoses and the purines, adenine and guanine, as well as the pyrimidines such as cytosine, uridine and thiamine, must have accumulated and combined into rudimentary ribonucleic and desoxyribonucleic acids.

In this period of chemical evolution, molecules were formed with or without the help of chance catalysts and were as likely to be of one optical configuration as another. A general principle is of importance here. It can be conveniently called the Chemical Valve Effect. Thus reaction rate control can be achieved through catalytic build up of a steady state concentration of an enzyme for a second reaction. If the compound built up is a powerful enzyme for a second important reaction, we have, as we shall see, a machine for altering biological history. Ribonucleic acid and desoxyribonucleic acid are, of course, catalysts for the formation of enzymes and consequently are examples of this valve or gate effect. Even though a catalyst is without effect on the concentrations of a system at equilibrium, it may vastly change the concentration of one of the intermediates in a system in a non-equilibrium steady state. This is because at equilibrium every reaction is exactly balanced by its inverse and a catalyst cannot change this balance since it speeds up forward and back reactions equally. In contrast, a steady state is the balance reached between reactions which synthesize a compound and the reactions which decompose it. Often back reactions are of minor importance. Thus, if synthesizing reactions are speeded up, an intermediate's concentration may be greatly increased,

while the contrary effect accompanies catalytic speed up of decomposition reactions.

In the confused mass of chemical reactions which had accumulated after a billion or so years of chemical evolution, crude enzymes were taking shape, only to break up again. The rudimentary precursors of ribonucleic acids were likewise forming and disappearing. Even in the presence of these fragments of modern molecules the primitive ribonucleic acid with the help of the rudimentary enzymes tended to make replicas of itself. In addition, the ribonucleic acid acting as a template facilitated and so sped the formation of enzymes. As long as the self-replication of ribonucleic acid was not sufficiently rapid to replace the parent template before it disintegrated from the ever present destructive reactions, the concentration of ribonucleic acid remained at a low level. Since template concentrations remained at a modest level, molecular evolution proceeded at a correspondingly modest pace. But this situation had within it the same sort of instability which is present in explosive mixtures involving branching chains where conditions are operating, such as a temperature rise, to increase branching up to the explosion point. The explosion comes when conditions have progressed to the point where more than one free radical is formed for each free radical disappearing.

Now as the partially self-replicating ribonucleic acid grew longer and more perfect, the point was finally reached such that with the available enzymes the template could more than replace itself within its life span. At this instant within the space of 10^{-13} sec. (the lifetime of the activated complex in any chemical reaction) the world suddenly changed. The pools of water greatly increased in their concentrations of ribonucleic acid until they reached a new stationary level. The slow halting changes of ineffectively catalyzed chemical evolution had now come to a close with the explosive-like emergence of total self-replication of ribonucleic acid. This change to a self-replicating world ushered in the era of biological evolution. The new era was characterized by intense chemical activity catalyzed by the abundant enzymes synthesized on the self-replicating templates of ribonucleic acid. These replicas, together with better adapted mutants, sealed the fate of all possible competitors. The same catalytic powers of enzymes which speed up the synthesis of the self-replicating templates also speed up their destruction. This new chemical tempo immediately cuts down the concentration by cannibalism of all poorly catalyzed competitors such as the precursors of the optical isomer of the critical complex. Consequently, as soon as any really efficient self-replicating optically active template, which synthesizes its cooperating enzymes, goes critical in the way outlined above, it slams the door on its competitors, and what

was previously a racemic world suddenly becomes and remains optically active.

APPLICATION OF REACTION RATE THEORY

It has frequently been argued that a critical complex such as our theory requires is too complicated to have arisen within the framework of known physical and chemical laws. An examination of this situation apparently leads to a different conclusion.

The most probable rate of appearance of our critical complex like other reactions is governed by the well known theory of absolute reaction rates. As for any reaction, we can write,

$$dc/dt = c_1c_2 \cdots c_m\kappa(kT/h)e^{-(\Delta F^{\ddagger}/RT)} \qquad (1)$$

Here dc/dt is the most probable rate for the appearance of our critical complex; c_1 to c_n are the concentrations of the respective reactants; κ is the transmission coefficient which we may here suppose to be unity; kT/h, which has the value 5.6×10^{12}, at 300° absolute is the frequency of reaction of the activated complex whose concentration is governed by the equilibrium constant

$$K\ddagger = e^{-(\Delta F^{\ddagger}/RT)}$$

$\Delta F\ddagger$ is, of course, the free energy of activation and k, T, h and R are the Boltzmann constant, the absolute temperature, Planck's constant and the gas constant, respectively.

Equation 1 may for our purpose be more conveniently written as,

$$dn/dt = n_1c_2 \cdots c_m(kT/h)e^{-(\Delta F^{\ddagger}/RT)}. \qquad (2)$$

Here we have simply changed from the concentrations, c_1 , of this reactant to the total number of such reactants, n_1 along with the necessary accompanying change over from dc/dt to the total number of critical complexes formed per second, dn/dt. Now in discussing *2* we will be obliged to introduce very crude estimates. These will, however, illustrate the kind of additional information required and, therefore, what experiments should be done, as well as the degree of overall reasonableness of the critical complex theory. We estimate all concentrations at 1/1000 molal. We assume next that m reactants join in a line to form the critical complex. Of the m-1 bonds so formed, we assume m-2 contribute factors of 1/100th each to $K\ddagger$ and that the final bond contributes the factor $e^{-(30{,}000/RT)}$ to $K\ddagger$ as the last bond passes over the potential barrier corresponding to the activated complex. A factor of 1/100th for a bond corresponds to a contribution to $\Delta F^{\ddagger}$ of +2.6 kilo calories, which is about the degree of instability assigned peptide bonds, for example (6). Contact stabilization by

adsorption of a molecule onto the two atoms forming an unstable bond could change an instability corresponding even to more positive free energies than +2.6 kilo calories into a stable negative contribution to the free energy. Our values, nonetheless, seem reasonable. We now must calculate the total number of reactants of the type n_1, whose concentration like the other reactants we suppose to be 1/1000th molal. To make the calculation we suppose that there were enough pools to average 10^6 liters per square mile over the whole earth's surface. This gives,

$$n_1 = \pi \times 8000^2 \times 10^6 \times (1/1000)\ 6.02 \times 10^{23} = 1.2 \times 10^{45} \text{ molecules.}$$

We next suppose conditions yielding the above average values continued over a period of a billion years. This gives

$$dn_1/dt = 1/(10^9 \times 365 \times 24 \times 60 \times 60) = (3.2 \times 10^{16})^{-1}.$$

Substituting these quantities into *equation 2*, we have

$$(3.2 \times 10^{16})^{-1} = 1.2 \times 10^{45}(1/1000)^{m-1}$$

$$5.6 \times 10^{12}(1/100)^{m-2}e^{-30{,}000/(2 \times 300)} \qquad (3)$$

Equation 3 leads to m = 12 for the number of reactants combining to form the critical complex. Now the value, m − 1 = 11, for the calculated number of bonds that are made in assembling the critical complex from reactants seems about right.

Undoubtedly, as we gather more information, we will want to modify the values of the various factors in our calculation of m, but the general theory of a critical complex operating as outlined is almost a mathematical necessity if we are to explain, within the framework of known physical and chemical principles, the overwhelming abundance in all species of the *l*-amino acids.

It is interesting that our theory, as expressed in *equation 2*, could be tested. For example, one might be able to arrange conditions so that our critical complex might appear in a reasonable time. Thus, if n_1 were taken as only a mole of molecules, i.e. 6×10^{23} instead of 1.2×10^{45}, and all concentrations of reactants were increased from 1/1000th to ½ molal, spontaneous generation of our critical complex in accord with the other values in *3*, using m = 12, would be speeded up by a factor of 4×10^{10}, or it should occur about every nine days. It would be interesting to try to find the proper concentrations of racemic molecules such that optical activity accompanied as it would be by a great increase in viscosity would, after an interval, suddenly appear spontaneously in a sterile system.

When one considers the number of molecules n_1 that might occur from broken cells in a living body and the probable concentrations of the

various constituents, it is clear that one should not expect to encounter spontaneous generation of our critical complex arising from as many as 12 elementary compounds. On the other hand, mutations arising from the recombination of two or three fragments ought to be observable as they are. It is, of course, an extremely lively question to ascertain how changes in cell heredity typified by cancer occur. When does cancer come from the addition of some desoxyribonucleic acid from virus-like bodies to the chromosomes, and when does it come from mutation inside the cell? Rate theory typified by *equation 3* is applicable in either case and should be an important tool in such studies. It is especially interesting to continue examining fossils to find out whether the amino acids they contain are all of the *l* form. Chromatographic separations and micro polarimetry facilitate such investigations. It is exceedingly interesting to be certain whether or not the burst of activity from the first optically active self-replicating critical complex really succeeded in slamming the door immediately on all competitors. With optically active enzymes once established, their dynamic stability against Walden inversion arises from the fact that usually inversion of an amino acid ruins the enzyme containing it, and so fatally handicaps the afflicted organism in its race for survival (7).

Our general explanation of the emergence of optical activity of the enzyme amino acids would be equally cogent were the critical complex some such molecule as desoxyribonucleic acid or an enzyme rather than ribonucleic acid. We have preferred the notion that ribonucleic acid was the critical complex in biogenesis, thinking of it as the most primitive template for proteins because of its wide distribution throughout the cytoplasm. However, additional evidence on this point would be welcome (6, 8, 9).

FURTHER EXAMPLES OF CRITICAL COMPLEX THEORY

As we have seen, the emergence of optically active enzymes ushered in a new epoch. A similar turning point probably occurred in nuclear evolution. With the discovery of anti-electrons (positrons) and anti-protons, it is natural to visualize another universe where positive electrons rotate about negative nuclei. This anti-world should be exactly as stable as our own world. Since two such systems would be mutually destructive, it is natural to postulate that in the period when energy first condensed to form matter, the same general type of fluctuations which gave us an *l*-amino acid world gave us billions of years early ascendancy of our negative electron world over its competitor. Professor Carl J. Christensen called our attention to the parallelism in the arguments required to explain this nuclear case. Finally, the growth of optical enantiomorphs, as of

quartz and the tartrates, from racemic mixtures exemplifies fluctuations where one optical isomer by nucleation gains the ascendancy and so forms optically active crystals without, however, having the ability to effectively destroy competitors.

Our unfamiliarity with life in other worlds makes us unable to test our assumption that independent biogeneses should yield *l* and *d* worlds substantially randomly. Goult (10), examining 1008 quartz crystals gathered from six widely separated areas, found *d* and *l* quartz in the ratio 51.6 to 48.4. This approaches a random distribution and invites further experimental and theoretical study. Systems like tartrates where the molecules as well as the crystals are enantiomorphic will necessarily show very close to equal frequency of occurrence of crystal types.

SUMMARY

The problem of biogenesis is discussed with reference to reaction rate theory and the significance of optically active catalytic systems in the living world. The facts that molecular systems in living organisms are optically active, that different amino acids within an organism are configurationally related, and that the various amino acids from different species of organisms are likewise related, are indicative of a common origin in the remote past, from a single optically active template, probably a ribonucleic acid molecule of greater length and configurational perfection than had previously occurred during some billion years of chemical evolution. From reaction rate theory, it follows that the template was formed in 10^{-13} sec., the lifetime of the activated complex in any chemical reaction. At this instant the world suddenly "went critical"; replication of the template and the crude enzymes involved in its formation now occurred within a shorter time than their own life spans. Simultaneously, the door was slammed on competitors with a different type of optical activity, and biological evolution began. An analogous event on some other world might as easily have given rise to life characterized by the *d*- rather than the *l*- type of optical isomer known in our world, the two types having exactly the same chemical reactivity. In nuclear evolution, an analogous turning point probably also occurred, so that it is natural to visualize some other universe where positive electrons rotate about negative nuclei, in contrast to the universe we know.

Reaction rate theory, together with crude estimates of reactant concentrations, leads to a reasonable rate of appearance, at 300° absolute, of the critical complex in biogenesis, according to known physical and chemical laws. With much greater concentrations, conditions could possibly be found whereby optical activity would spontaneously arise in a sterile system in a few days.

REFERENCES

1. Oparin, A. I. *The Origin of Life* (tr. by S. Margulis). New York: Macmillan, 1938.
2. Blum, H. F. *Times Arrow and Evolution* (2nd ed). Princeton, N. J.: Princeton, 1935.
3. Calvin, Melvin. Chemical evolution and the origin of life. *Am. Scientist* 44: 248, 1956.
4. Fox, Sidney W. Evolution of protein molecules and thermal synthesis of biochemical substances. *Am. Scientist* 44: 347, 1956.
5. Urey, H. C. *The Planets*. New Haven: Yale, 1952.
6. Borsook, Henry. The biosynthesis of peptides and proteins. *J. Cell. & Comp. Physiol.*, 47 (Suppl. 1): 35, 1956.
7. Johnson, F. H., H. Eyring, and M. J. Polissar. *The Kinetic Basis of Molecular Biology*. New York: Wiley, 1954.
8. Dounce, A. L., et al. Nucleoproteins (round table discussion). *Ibid*, 47: 103, 1956.
9. Haurowitz, F. The mechanism of protein biosynthesis. *Proceedings of the Third International Congress of Biochemistry* (Brussels, 1955). New York: Acad. Press, 1956.
10. Goult, R. H. Frequency of twin type in quartz crystals. *Am. Mineralogist* 34.

STRUCTURAL FACTORS INVOLVED IN PROTEIN STABILITY

W. Kauzmann, *Frick Chemical Laboratory, Princeton University, Princeton, New Jersey*

Proteins are among the more temperature-sensitive components of living systems, so that a discussion of the effects of temperature on proteins is surely pertinent to the present symposium. The most important of these temperature effects is the phenomenon largely responsible for the thermal instability of proteins that is usually referred to as denaturation. The present paper will be concerned with some aspects of this phenomenon. Let us suppose that an investigator is fortunate enough to have isolated some new protein from a living system and that he has decided to study its stability.

What are some of the factors that this investigator will have to keep in mind in making such a study? He may be tempted to use the word 'denaturation' to describe his studies, and since the meaning of this word has caused some misunderstanding, it is perhaps well to devote a few paragraphs to it. The investigator will also have to decide on an experimental approach to the problem of stability, and he will presumably want to study the changes in some property that will tell him what is taking place inside the protein molecule when it denatures. A brief survey of some of the more useful methods available for studying changes in protein structure will therefore be given, special emphasis being placed on two particularly important and convenient methods. General reviews of the denaturation phenomenon have been given by Neurath *et al.* (24), Haurowitz (9), Anson (1, 2), Putnam (26), Kauzmann (15) and Lumry and Eyring (21). The significance of denaturation in various biological processes has been discussed by Johnson, Eyring and Polissar (14).

DEFINITIONS OF DENATURATION AND THE CONCEPT OF PROTEIN STABILITY

When they are heated, many globular proteins lose the properties that are responsible for the important role that they play in biological systems. They may become insoluble in water at their isoelectric points and, if they have any biological functions such as enzymatic activity, these functions may be lost. The heated protein is said to be 'denatured'. Denaturation in this sense (i.e. the loss of solubility in water or dilute salts at the isoelectric point and the loss of any biologically significant activity that the

protein may possess) may also be brought about by treatment with numerous other relatively mild chemical and physical agents.

It is natural to ask what changes in the structure of the protein molecule are responsible for the process of denaturation that has just been described. We know that proteins consist of long polypeptide chains that are coiled up in a reproducible fashion so that the surface of the molecule presents definite patterns of amino acid residues and peptide linkages to its environment. These patterns are believed to determine the characteristic properties of proteins. When the patterns are changed, the protein may lose these properties. Of course, one can always change the surface pattern of a protein by chemical alteration of its amino acid residues—e.g. by acetylating the free amino groups on the lysine residues, by iodinating the tyrosine rings, by letting the sulfhydryl groups react with silver or mercury or by changing the state of ionization of a carboxyl or amino group. Chemical changes of this kind are not, however, usually considered to be examples of denaturation, even though they may lead to a loss of activity. The kinds of structural changes that might be considered to lead to denaturation include the following: *1*) association of two or more protein molecules into larger units within which essential portions of the surface pattern are buried; *2*) dissociation of the protein molecule into smaller units, thus fragmenting the essential patterns; *3*) changes in the way in which the polypeptide chain is folded, without any change in the molecular weight.

There is much evidence that the process of denaturation described in the first paragraph of this paper frequently results from the third type of change. It is therefore natural to identify the process of denaturation with changes resulting from an alteration in the manner in which the polypeptide chain is folded. In some instances, however, it is possible that loss of biological activity may result from dissociation into sub-units without any changes in the manner of folding of the polypeptide chains within the sub-units. This may be the case, for instance, in the inactivation of tobacco mosaic virus by urea (19). There is little evidence, however, that association without accompanying changes in the state of folding ever results in a loss of activity.

In the paragraphs immediately preceding there are to be found the roots of two entirely different possible definitions of the word 'denaturation.' The definition that would result from the first paragraph of this section of the paper is based on directly observable experimental criteria of solubility and biological activity. The definition derived from the second paragraph of this section would be phrased in terms of changes in the structure of the protein molecule that may be deduced only rather indirectly from experimental observations. Definitions derived from each point of view have been widely used by protein chemists. Each kind of

definition has its advantages and its disadvantages. A definition that is based directly on simple experimental criteria has the apparent advantage that it is expressed in operational terms, so that in using it the experimenter will not be likely to have much difficulty in concluding whether or not denaturation has occurred under a particular set of conditions. A definition in terms of changes in structure may be difficult to apply because there are not many methods of detecting changes in the state of folding of the polypeptide chain—especially if these changes are slight. Therefore the definition in terms of loss of solubility and activity is likely to appeal to the man in the laboratory. On the other hand, a definition in structural terms focuses attention on the essential changes that take place in the protein molecule and therefore comes to grips with a more fundamental aspect of the problem. Furthermore, the existence of reversible denaturation may seriously limit the 'experimental' definition in an undesirable way, as we shall see.

The widespread use of these two very different and equally legitimate types of definition has lead to confusion and has made some workers suggest that the term 'denaturation' be discarded altogether. The word is so widely used, however, and there is such a need for some term to designate the phenomenon, that it is not likely to be dropped. If we recognize that the term has several legitimate meanings, and if in using it we always make it clear just which meaning is intended, much of the confusion that the word sometimes seems to cause should disappear. Besides, if we are going to discard the word, a new word will have to be invented. This hardly seems advisable at the present time because, as Anson has said (1), we do not yet know enough about the phenomenon to be able to define precisely the word that we use to describe it. It seems much simpler to continue to use the word with the dual meanings that have been given to it in the past, always making clear which meaning is intended.

It might seem unnecessary to devote so much space to this matter of defining a term that almost everyone thinks he understands, especially when no unique definition has been finally decided upon. The concept of denaturation plays a very important role in discussions of the stability of proteins, however, and we can come to very different conclusions, depending on how we choose to define the phenomenon. For instance, serum albumin can be exposed to pH 2 or to 8M urea for long periods without losing its solubility in water at its isoelectric point. Exposure of ovalbumin to the same conditions leads to a rapid loss of its solubility at the isoelectric point. Therefore, according to the 'experimental' definition of denaturation, we should conclude that ovalbumin is much easier to denature than is serum albumin. On examining the optical rotations and the intrinsic viscosities of the two proteins while they are exposed to the denaturing

conditions, however, it is found that serum albumin undergoes an instantaneous change to a much less compactly folded state as soon as it is exposed to acid or urea, whereas ovalbumin undergoes similar changes much less rapidly. If we define denaturation in terms of changes in the way the polypeptide chain is folded, we must conclude that ovalbumin is more resistant than serum albumin to denaturation by acids and by urea. The reluctance of serum albumin to lose its solubility in water at the isoelectric point is merely the result of the ability of its polypeptide chain to refold into a state closely similar to (if not identical with) that of the native protein as soon as the denaturing agent (acid or urea) is removed. It is obvious that if we were to use solubility in water at the isoelectric point as the sole criterion of denaturation, we should overlook some important instances in which proteins temporarily lose the structures that characterize the native form. It is clearly desirable that in setting up experimental criteria for denaturation, these criteria should be applicable while the protein is exposed to the denaturing agent. If this is not done, and if the protein can change its structure reversibly, it is possible to gain a completely false impression of the lability of the native structure in the presence of denaturing agents.

METHODS AVAILABLE FOR OBSERVING STRUCTURAL CHANGES IN PROTEINS DURING DENATURATION

If we adopt the more fundamental approach to the phenomenon of denaturation and choose as the criterion of denaturation a change in the state of folding of the polypeptide chain rather than loss of solubility or biological activity, then suitable experimental methods of detecting these changes in folding must be available. In order to avoid the uncertainties caused by reversible denaturation, these methods should be usable when the protein is in the presence of the denaturing agent.

The properties of proteins that change when the polypeptide chain changes its state of folding fall into two general classes. One class of property depends on the over-all geometrical shape of the molecule and only indirectly, if at all, on the chemical composition and the precise spatial relationships of the different parts of the polypeptide chain to their immediate neighboring parts. The other class of property depends on the short range interactions of each part of the chain with other parts that are close to it. For brevity the two classes may be called *shape properties* and *short-range properties*. The distinction between the two classes may best be clarified by listing the more important members of each class.

Shape Properties (Many of the following properties depend also on the molecular size, so that an independent measurement of the molecular weight may be necessary in judging the structural significance of a change

in the property): *1)* Solution viscosity. *2)* Friction ratio (as determined from the molecular weight and the diffusion constant). *3)* Rotatory diffusion constants (as determined from flow birefringence, the decay of the Kerr effect, and the depolarization of the fluorescence from adsorbed or coupled dye molecules). *4)* Dissymmetry of light scattering. *5)* Low angle x-ray scattering. *6)* The second virial coefficient of the osmotic pressure (or light scattering) under conditions that suppress electrostatic interactions between molecules (i.e. moderate salt concentrations).

Short-range Properties: *1)* The infra-red absorption spectrum. *2)* The visible and ultra-violet absorption spectrum. *3)* The partial specific volume. (Small changes during denaturation are readily measured in dilatometers.) *4)* The refractive index increment (small changes during denaturation are readily measured by means of an interferometer.[1]) *5)* Optical rotation. *6)* Dissociation constants of acidic and basic groups. (Changes in dissociation constants result in the release or absorption of hydrogen ions which can be followed readily as a function of time by means of recording pH-stats, such as those of Jacobsen and Léonis (10) and of Neilands and Cannon (23).) *7)* Changes in the reactivities of other groups, such as the sulfhydryl group. (The ease of exchange of deuterium atoms with hydrogen atoms, which has been studied recently by Linderstrom-Lang and co-workers (20), is another example of a change in reactivity resulting from denaturation.)

Detailed reviews of the experimental measurement of shape properties, as well as of their interpretation, have been given by Edsall (5), Gutfreund (8) and Sadron (28).

Since the structural information provided by the two classes of properties differs in its basic character, both classes are useful in detecting and understanding the phenomenon of denaturation. Wherever possible, studies of denaturation ought to include an investigation of the changes in at least one property from each of the two classes.

Unfortunately many of the properties that have been listed above are not particularly well suited for more routine investigations of denaturation, such as one would be likely to conduct in a preliminary study of the stability of a newly isolated protein. For instance, the measurement of

[1] In the case of the urea denaturation of ovalbumin it has been found (17) that changes in the refractive index can be accounted for entirely in terms of the change in volume that accompanies denaturation, assuming that the specific refractivity of the solution, $r = (n^2 - 1)/(n^2 + 2)d$, is a constant, where n is the refractive index and d is the density of the solution. The fact that r is constant during denaturation indicates that the polarizability of the protein molecule does not change during denaturation, at least in the case of the urea denaturation of ovalbumin. Thus it appears that the interferometer measures the same thing as the dilatometer does.

diffusion constants requires rather long times and is subject to uncertainties when the protein is not homogeneous. Sedimentation constants do not tell anything unequivocal about the shape of a protein because they depend on the molecular weight as well as on the shape; they are dependable indications of unfolding only if an independent measurement of the molecular weight has been made under the same conditions as those present in the sedimentation run. Flow birefringence is observable with equipment that is now available only if the particles are quite long or if the viscosity of the solvent has been raised substantially by adding large amounts of such substances as sucrose or glycerol—which may have important effects of their own on the denaturation process. Measurements of the dissymmetry of light scattering are not useful in determining particle shapes unless the particle has at least one dimension of the order of 1000 Å.; this makes the method unsuitable for proteins whose molecular weight is smaller than about 100,000 (which eliminates the method for study of such common proteins as ovalbumin, serum albumin and insulin in their denatured, but unaggregated state). Low angle x-ray scattering requires apparatus that is not available in most laboratories. Depolarization of fluorescent light requires studies with each protein-dye complex of the lifetime of the excited state of the fluorescent molecule that is used. The measurement of the infra-red absorption of proteins in solutions containing water encounters difficulties because of the strong absorption of water over the most readily accessible range of infra-red wave lengths. Except with the chromoproteins such as hemoglobin and cytochrome *c*, denaturation does not seem to have much effect on the visible and ultraviolet absorption spectrum of proteins. (An important exception is the spectral change that occurs when some proteins are denatured at alkaline pH, which is brought about indirectly by a shift in the dissociation constant of the hydroxyl group of tyrosine, refs 3, 31.) Changes with time of the partial specific volume and refractive index are easy to measure at room temperature, but because of the need for very close temperature control, they are not so easy to follow at other temperatures. Furthermore, if the protein unfolds very rapidly on exposure to the denaturing agent, it may be difficult to separate the changes that are caused by denaturation from the changes brought about by the addition of the denaturing agent to the solvent.

Fortunately two properties, one from each of the two classes mentioned above, are easily measured with apparatus that is either easily constructed or is readily available in most chemical and biochemical laboratories. These two properties are the viscosity and the optical rotation. Largely because of the ease with which they can be measured they are frequently

used in studies of denaturation at the present time.[2] It is therefore worth devoting some space to a discussion of the structural significance of changes in these two properties. What can we infer if the viscosity and the optical rotation of a protein solution change their values under the influence of some denaturing agent?

INTERPRETATION OF CHANGES IN SOLUTION VISCOSITY DURING PROTEIN DENATURATION

It has been usual in the past, and it continues to be the fashion, to interpret the viscosity increment of protein solutions in terms of models that are rigid ellipsoids (see, for instance, Scheraga and Mandelkern, 30). This seems to be a reasonable procedure when it is applied to a protein in a relatively compact folded state, such as that assumed by the native protein, and also by the denatured protein in a poor solvent. On the other hand, if the denatured protein is dissolved in a good solvent which interacts strongly with a large fraction of the groups that occur along the polypeptide chain, then very viscous solutions are obtained, indicating that the molecule has unfolded to a much more extended form. (This is probably the case for strong urea solutions of most proteins, for instance.)

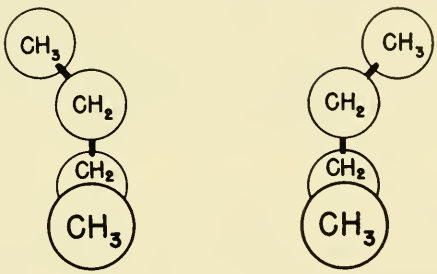


FIG. 1

In such solvents a more reasonable model than the rigid ellipsoid is provided by the randomly coiled chain that is so familiar to the polymer chemist (chap. 10 of Flory, 6). If the secondary forces (van der Waals forces, hydrogen bonds, etc.) that operate between different segments of the polymer chain are small compared with the thermal energy, so that

[2] The measurement of the effect of a protein on the viscosity of a solution at elevated temperatures is not too easy to accomplish because of the need for fairly good temperature control. This need can be avoided by using reasonably well stirred baths containing two viscosimeters, one containing pure solvent and the other containing the protein solution; outflow times are measured concurrently on the two viscosimeters.

the chain shows no tendency to fold up or to crystallize, then it is known that polymer chains tend to squirm about, assuming all possible shapes in much the same manner that a piece of spaghetti does when it is tossed into a pot of boiling water, or a string of beads does when it is tossed into the air. This writhing chain is what is known as the random coil. Polymer chains take on the multitude of shapes that characterize the random coil for the same reason that gas molecules tend to fill up a room—the random coil is a state of high entropy.

Now it is important to note that the three principal hydrodynamic properties of a random coil (that is, the viscosity increment, the rotatory diffusion constant and the friction ratio) cannot be duplicated by any single equivalent ellipsoid (see footnote 41 of Scheraga and Mandelkern, 30). It is probably also true that any other shape that differs markedly from an ellipsoid—such as a hoop, a helix, a spiral or an elbow—would also not have hydrodynamic properties that are exactly the same as those produced by any single ellipsoid. The preoccupation that protein chemists have for rigid ellipsoids would therefore seem to be unwise when applied to proteins under certain conditions. This is especially true because the theory of the hydrodynamic properties of random coils has been thoroughly developed (see Flory, 6, chap. 14). A preliminary attempt to interpret the hydrodynamic properties of some denatured proteins has been given (15), but more experimental work ought to be done from this point of view. A great deal could be learned about such details as the intramolecular cross-linking of protein molecules in this way.

OPTICAL ROTATORY POWER AND PROTEIN DENATURATION

The theory of the relationship between optical rotatory power and molecular structure has not yet been worked out in detail, but certain general principles are known which should be kept in mind by anyone using this property to study proteins. Many people seem to have the misconception that optical rotatory power somehow resides in the asymmetric carbon atom. This is quite wrong. According to Pasteur, the sole requirement that must be fulfilled in order that a substance be able to rotate the plane of polarization of light is that the molecules of which the substance is composed must not be superimposible on their mirror images. All of the modern theories of optical rotatory power are in complete harmony with Pasteur's rule. This means, for instance, that the following two conformational isomers of the simple molecule, butane (which contains no asymmetric carbon atom), would show optical activity if they could be separated from each other:

The only reason they have not been separated in the laboratory is that there is a very low energy barrier between them, so that they change into one another (or 'racemize') much too rapidly for them to be isolated in the two pure forms. The property of the asymmetric carbon atom that makes it so important in giving rise to optical rotatory power is merely that it makes it so difficult for the mirror image forms of a substance to come into equilibrium. In chemical compounds containing asymmetric carbon atoms, chemical bonds have to be broken in order to pass from one form of the molecule to its mirror image form, whereas in a molecule such as butane one has only to rotate the two halves of the molecule about the central carbon-carbon bond. Thus the asymmetric carbon atom is merely a means of stabilizing optical isomers. Organic chemists have found other means of accomplishing this in certain favorable cases (e.g. the use of steric hindrance in ortho-substituted diphenyls).

Thus the asymmetric carbon atom provides the geometrical condition necessary for optical rotation but does not itself necessarily interact directly with the light wave. What is it, then, that determines the actual amount of the rotation of the plane of polarization? All of the physical theories agree that the phenomenon arises from the electronic motions that

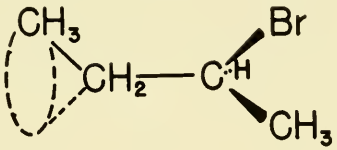


Fig. 2

are induced in the chromophoric groups of the molecules when light shines on them. These electronic motions are the same as those that are responsible for the ultra-violet and visible absorption spectra of molecules; in most cases the largest contribution to the optical rotation comes from the motions that belong to the absorption band whose wave length is closest to the wave length of the light that is used to measure the rotation. For instance, the sign of the optical rotation of visible light by camphor is determined by the electronic motions that give rise to the weak carbonyl absorption band of camphor at 2900 Å.[3]

Because of the dissymetric environment of the chromophores, the elec-

[3] The electrons on asymmetric carbon atoms are almost invariably associated with absorption bands that lie in the far ultra-violet, well below 2000 Å. Furthermore, there are usually many other electrons in optically active organic molecules that have similar absorption bands. Therefore the direct contribution of the asymmetric carbon atom itself to the magnitude of the optical rotation is probably almost always small.

trons in optically active molecules tend to move in slightly skewed paths when light shines on them. It is this skewness that causes the rotation of the plane of polarization of light. Unfortunately the mechanism by which the skewness does this is too complicated to be discussed here, but it is not necessary to know the mechanism in order to understand the discussion that follows. (See ref. 16 for a detailed discussion of the mechanism.)

When the positions of the groups in the immediate vicinity of a chromophoric group are altered, the skewness that they cause in the currents induced by the light is changed and the magnitude of the optical rotation changes. It turns out that these effects are so large that the optical rotation is very much more sensitive than most properties to rather subtle modifications of molecular structure. For instance, in a molecule such as secondary butyl bromide, there is every reason to believe that the magnitude (and even the sign) of the optical rotation will change drastically when the ethyl group in the left half of the molecule (fig. 2) is turned about the central carbon-carbon bond. Other properties, such as the refractive index, polarizability and infra-red and ultra-violet spectra, would hardly be affected by this slight modification in the structure. Such structural changes, which involve a twisting of the molecular framework without making any changes in the chemical composition and without breaking any chemical bonds, are called changes in *conformation*. (The conformation of a molecule is also sometimes called its *constellation*. The word *configuration* is also frequently used by protein and polymer chemists as a synonym for conformation, but this word has long been used by organic chemists with reference to the spatial arrangement of the groups attached to the asymmetric carbon atom.)

Whenever a substance undergoes a large change in optical rotation without any appreciable chemical modification of its structure, we can be sure that there has been a change in the molecular conformation. It is therefore highly significant that when proteins are denatured, they usually undergo large changes in optical rotation. The magnitudes of the changes will be discussed in more detail below, but the mere fact that the changes are as large as they are offers strong support for the concept that denaturation involves changes in the manner in which the protein molecule is folded.

Unfortunately our understanding of the details of the relationship between molecular structure and the magnitude of the optical activity[4] is still in a rather primitive state. Therefore, if the optical activity is observed

[4] In this paragraph the term 'optical rotation' will be replaced by the equivalent but less precise designation 'optical activity'. This helps to avoid confusion between the rotation of groups about single bonds and the rotation of the plane of polarization of polarized light.

to change in an experiment, it is not yet possible to infer very much from the magnitude of this change about the nature of the underlying change in conformation. All the principal modern theories of optical activity agree, however, that the following rule ought to be true: if all of the groups attached to the asymmetric carbon atoms are free to rotate about the bonds that hold them to the asymmetric carbon atoms, then a relatively small optical activity should be observed. To illustrate this, consider the secondary butyl alcohol molecule,

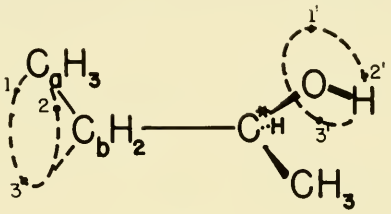


FIG. 3

If the ethyl group, C_aH_3—C_bH_2—, can orient freely about the C_b—C* bond, and if the OH group can orient freely about the C*—O bond, then according to the rule the optical rotation of the molecule ought to be small.[5] This rule is based on an interesting internal compensation effect that is described elsewhere (18). The available experimental evidence indicates that if there is free orientation about bonds, then values of $[\alpha]_D$ of less than 10° will be observed in typical organic molecules that do not have absorption bands in the visible or near ultra-violet. Any restriction of the ability of groups to orient freely about these bonds will usually produce much larger optical activities. Such restrictions can be expected if the asymmetric carbon atoms occur in small rings, or if the groups attached to the asymmetric carbon atoms are bulky.

In the case of restriction by bulky groups, the magnitude of the optical activity almost always decreases when the temperature is raised because higher temperatures lead to greater freedom of orientation. With ring compounds an increase in temperature cannot cause completely free orientation about single bonds without rupturing some of the bonds in the ring, so the temperature coefficient of the magnitude of the optical activity is as often positive as it is negative. Of course, it is possible to have

[5] Strictly speaking, in order that a small optical activity be observed, it is only necessary that C_a occupy with equal frequency the three symmetrically disposed positions, 1, 2 and 3 indicated in the above sketch, and that the hydrogen atom on the hydroxyl group occupy with equal frequency the three symmetrically disposed positions 1′, 2′ and 3′. The term, 'free orientation about a bond,' will be understood to mean the existence of equal populations in several such symmetrical positions.

an optical activity that is small in an occasional compound containing groups whose movements are restricted. On the other hand, the optical activity should always be small in compounds containing groups that orient freely about all of the single bonds that are attached to asymmetric carbon atoms. Thus, a small optical activity is a necessary basis, but not a sufficient one, for concluding that free orientation exists in a molecule containing asymmetric carbon atoms. If $[\alpha]_D$ is much greater than 10° for organic molecules that do not have absorption bands in the visible or near ultra-violet, then we can also conclude that the orientation about single bonds is restricted.

Most native proteins have values of $[\alpha]_D$ that fall between −20° and −60°, and on denaturation $[\alpha]_D$ invariably becomes more negative. In strong urea solutions values as large as −120° may be reached at low temperatures, but heat denaturation in the absence of urea usually leads to values of −70° to −80°. (For detailed data see especially the papers of Jirgensons, 11–13, and the following important paper by Schellman, 29.)

The fact that native proteins have optical rotations of the same sign and similar order of magnitude is an indication that some basic structural resemblance between them must exist. One is tempted to believe that this similarity may reside in the helical structure proposed by Pauling and Corey (25), but recent work with synthetic polypeptides by Doty and his co-workers indicate that the helix itself is probably dextrorotatory (4). Yang and Doty (32) have made the very plausible suggestion that the negative sign of the optical rotation of native proteins is a result of the partial state of imperfect folding that exists in some degree in all native proteins. That is, sections of the polypeptide chain may be folded into helices, but intervening portions have the disorganized structure—and also the greater levorotation—that characterize the denatured protein.

The fact that denatured proteins have relatively large optical rotations is an indication that although the molecule may have an over-all shape resembling that of a random coil (which implies a certain amount of flexibility in the chain), nevertheless a considerable amount of steric hindrance must be operative, preventing completely free orientation about the bonds attached to the asymmetric carbon atoms. Short sections of the polypeptide chain must therefore be relatively stiff, even in the denatured molecule.

The magnitude of the optical rotation of denatured proteins in urea solutions decreases markedly with increasing temperature, just as we should expect if freedom of orientation increased with increasing temperature. The temperature coefficients of the optical rotations of denatured proteins in water are surprisingly small, however.

There is an appreciable change in the optical rotation when chymo-

trypsinogen is converted to chymotrypsin, $[\alpha]_D$ being $-77°$ for the zymogen and $-66°$ for the enzyme (29). This change has recently been studied in some detail by Rupley, Dreyer and Neurath (27), who find that the change in optical rotation parallels the appearance of enzymatic activity. Since no alpha amino end group is detectable in chymotrypsinogen, whereas one is present in chymotrypsin, it is believed that the zymogen is a large cyclic peptide and that one stage of the activation process entails the opening of the polypeptide ring. It is natural to suspect that the presence of the closed ring in the zymogen keeps the polypeptide chain from folding as completely into a helix as it might otherwise if the ring were opened. The rupture of one peptide link during the conversion to the enzyme would then make it possible for more of the chain to fold into a helix, and since the helix appears to be dextrorotatory, this would lead to the observed decrease in the levorotation on activation of the enzyme.

The large number of cystine disulfide crosslinkages in the serum albumin molecule appears to interfere in a similar fashion with the development of helices. Earlier work had shown (7), through the effect of reducing agents on the viscosity of denatured serum albumin in urea, that the two halves of many, if not all, of the cystine residues are not situated at adjacent positions along the polypeptide chain, so that the molecule is rather extensively cross-linked. Unless these cross links were especially favorably located along the chain, they would be expected to interfere with the normal development of a helical structure within the serum albumin molecule. That such interferences actually exist is indicated by the relatively large optical rotation of serum albumin ($[\alpha]_D = -60°$) and by the great ease with which it may be denatured in urea. It is very interesting, therefore, that Markus and Karush (22) have recently observed that when the disulfide groups of serum albumin are reduced (detergent must be present in order to make the disulfide groups sufficiently reactive), a considerable decrease in the levorotation takes place.

It is tempting to suppose that proteins can exist in two extreme states of folding: an 'ultra-native' form which would have completely developed helices and which would have a relatively small levorotation, or perhaps even a small dextrorotation; and a denatured form that contains no helices at all and is rather strongly levorotatory at ordinary temperatures. In native protein molecules as they exist in living systems, the polypeptide chain exists in both states of folding, the relative amounts that are present being different in different proteins. The magnitude of the levorotation would then be a measure of the amount of the denatured type of folding present in the molecule. Thus serum albumin ($[\alpha]_D = -60°$) would contain a relatively large amount of the denatured type of folding, whereas ovalbumin ($[\alpha]_D = -30°$) and beta-lactoglobulin ($[\alpha]_D = -26°$)

would contain more of the native type of folding. The relative amounts of the two forms would presumably vary as the temperature, the solvent and the extent of intramolecular cross-linking were varied.

It should be strongly emphasized that this attractive hypothesis must be used with a great deal of caution. The polypeptide chain of proteins, with the wide variety of side chains that it has dangling from it, may very well be able to fold in states other than the two that have been postulated above. Optical rotatory power provides us with but a single number. Useful though this number may be in characterizing the state of the molecule, it could easily lead to a serious oversimplification of our interpretation of the changes in protein structure that occur on denaturation under different conditions. One shudders to think of the oversights that would be possible if, for instance, two completely different states of folding happened to have the same optical rotation.

SUMMARY

One of the principal factors determining the behavior of living organisms at high temperatures is the stability of their proteins, and any discussion of the effects of temperature on protein stability will usually invoke the word 'denaturation.' This word has long been used to describe the loss of activity and solubility that results from exposure to high temperature, but more recently it has been defined in more fundamental terms, involving reversible and irreversible structural changes peculiar to proteins. Both meanings of the word are useful, but confusion has resulted when the existence of two meanings has not been recognized.

Two complementary types of experimental measurement are available for detecting the structural changes that accompany protein denaturation. One type of property is sensitive to the over-all shape of the molecule and is illustrated by the solution viscosity, the rotational diffusion rate and dissymmetry in the scattering of light. The other type of property is determined by short-range interactions between different parts of the molecule and is illustrated by infra-red and ultra-violet spectra, optical rotatory power and the chemical reactivity of groups in the molecule.

Studies of the structural changes that occur in proteins during denaturation should, if possible, be made using at least one property from each of the two types in order to give a maximum amount of structural information with a given amount of experimental effort. The viscosity and the optical rotatory power are especially convenient for this purpose. Structural changes that lead to a change in viscosity are usually interpreted in terms of a model consisting of a rigid ellipsoid, but the random coil model is more suitable for some conditions and it is not hydrodynamically

equivalent to any ellipsoid. The changes in optical rotation that occur on denaturation are often attributed vaguely to changes in the contributions of asymmetric carbon atoms present in the protein molecule. This terminology is inappropriate because the magnitude of the optical rotation depends only very indirectly on the presence of asymmetrical carbon atoms in a molecule. The important factor is the vicinal actions of groups on each other. These interactions change whenever the relative spatial positions of groups are changed. Therefore the optical rotation of a protein will change whenever the polypeptide chain is twisted into a new conformation even though no change may have taken place in the asymmetric carbon atoms themselves. Some interesting recent studies of the viscosity and optical rotation changes on denaturation are discussed.

REFERENCES

1. Anson, M. L. The Denaturation of Proteins. In: *Les Protéines, Rapports et Discussions*. R. Stoops (editor). Brussels: Institut International de Chimie Solvay, 1953, pp. 201–246.
2. Anson, M. L. Protein denaturation and properties of protein groups. *Advances in Protein Chem.* 2: 361–386, 1945.
3. Crammer, J. L. and A. Neuberger. The state of tyrosine in egg albumin and in insulin as determined by spectrophotometric titration. *Biochem J.* 37: 302–310, 1943.
4. Doty, P. and J. T. Yang. Polypeptides VII. Poly-gamma-benzyl-L-glutamate: the helix-coil transition in solution. *J. Am. Chem. Soc.* 78: 498–500, 1956.
5. Edsall, J. T. The Size, Shape and Hydration of Protein Molecules. In: *The Proteins*, H. Neurath and K. Bailey (editors). New York: Acad. Press, 1953, vol. IB, pp. 549–726.
6. Flory, P. J. Principles of Polymer Chemistry. Ithaca, N. Y.: Cornell Univ. Press, 1953.
7. Frensdorff, H. K., M. T. Watson and W. Kauzmann. The kinetics of protein denaturation. V. The viscosity of urea solutions of serum albumin. *J. Am. Chem. Soc.* 75: 5167–5172, 1953.
8. Gutfreund, H. The Properties of Solutions of Large Molecules. In: *Progress in Biophysics*, J. A. V. Butler and J. T. Randall (editors). New York: Acad. Press, 1950, vol. 1, pp. 1–38.
9. Haurowitz, F. *The Chemistry and Biology of the Proteins*. New York: Acad. Press, 1950.
10. Jacobsen, C. F. and J. Léonis. A recording auto-titrator. *Compt. rend. trav. lab. Carlsberg, Sér. chim.* 27: 333–339, 1951.
11. Jirgensons, B. Denaturation of plant proteins. III. Optical rotation, viscosity and reducing capacity of denatured proteins. *J. Polymer Sci.* 5: 179–189, 1950.
12. Jirgensons, B. Optical rotation and viscosity of native and denatured proteins. I. Influence of pH, and the stability of various proteins toward several denaturing agents. *Arch. Biochem.* 39: 261–270, 1952.
13. Jirgensons, B. Optical rotation and viscosity of native and denatured proteins. II. Influence of temperature and concentration. *Arch. Biochem.* 41: 333–344, 1952.
14. Johnson, F. H., H. Eyring and M. J. Polissar. *The Kinetic Basis of Molecular Biology*. New York: Wiley, 1954.

15. Kauzmann, W. Denaturation of Proteins and Enzymes. In: *The Mechanism of Enzyme Action*. W. D. McElroy and B. Glass (editors). Baltimore: Johis Hopkins Press, 1954, pp. 70–110.
16. Kauzmann, W. *Quantum Chemistry: an Introduction*. New York: Acad. Press, 1957.
17. Kauzmann, W. Unpublished observations.
18. Kauzmann, W. and H. Eyring. The effect of rotation of groups about bonds on optical rotatory power. *J. Chem. Phys.* 9: 41–53, 1941.
19. Lauffer, M. The denaturation of tobacco mosaic virus by urea. II. Kinetic aspects. *J. Am. Chem. Soc.* 65: 1793–1802, 1943.
20. Linderstrøm-Lang, K. Deuterium Exchange between Peptides and Water. In: *Peptide Chemistry*, D. H. Hey (editor). Special Publication No. 2, Chemical Society, London, 1955.
21. Lumry, R. and H. Eyring. Conformation changes in proteins. *J. Phys. Chem.* 58: 110–120, 1954.
22. Markus, G. and F. Karush. *J. Am. Chem. Soc.* 79: 134–139, 1957.
23. Neilands, J. B. and M. D. Cannon. Automatic recording pH instrumentation. *Anal. Chem.* 27: 29–33, 1955.
24. Neurath, H., J. P. Greenstein, F. W. Putnam and J. O. Erickson. The chemistry of protein denaturation. *Chem. Rev.* 34: 157–266, 1944.
25. Pauling, L. The Configuration of Polypeptide Chains in Proteins. In: *Les Protéines, Rapports et Discussions*, R. Stoops (editor). Brussels: Institute Internationale de Chimie Solvay, 1953, pp. 63–69.
26. Putnam, F. W. Protein Denaturation. In: *The Proteins*, H. Neurath and K. Bailey (editors). New York: Acad. Press, 1953, vol. IB, pp. 807–892.
27. Rupley, J. A., W. J. Dreyer and H. Neurath. Structural changes in the activation of chymotrypsinogen. *Biochim. et biophys. acta* 18: 162–163, 1955.
28. Sadron, C. Methods of Determining the Form and Dimensions of Particles in Solution: A Critical Survey. In: *Progress in Biophysics*, J. A. V. Butler and J. T. Randall (editors). New York: Acad. Press, 1953, vol. 3, pp. 237–304.
29. Schellman, J. *Compt. rend. trav. lab. Carlsberg, Sér. chim.* In press.
30. Scheraga, H. A. and L. Mandelkern. Consideration of the hydrodynamic properties of proteins. *J. Am. Chem. Soc.* 75: 179–184, 1953.
31. Tanford, C. and G. L. Roberts. Phenolic hydroxyl ionization in proteins. I. Bovine serum albumin. *J. Am. Chem. Soc.* 74: 2509–2515, 1952.
32. Yang, J. T. and P. Doty. The optical rotatory dispersion of polypeptides and proteins in relation to configuration. *J. Am. Chem. Soc.* 79: 761–775, 1957.

EFFECTS OF TEMPERATURE, DIELECTRIC CONSTANT AND DIFFUSION RATES ON FORMATION OF THE INTERMEDIATE COMPOUND OF CATALASE AND HYDROGEN PEROXIDE

Eugene Ackerman, Greenville Kash Strother and Robert Lewis Berger,[1] *Physics Department, Pennsylvania State University, University Park, Pennsylvania*

Catalase is an enzyme, found widely distributed throughout living matter. The speed with which this heme protein decomposes hydrogen peroxide led to its early discovery. However, the details of its action were only elucidated since the last war, due primarily to the studies of Chance and his co-workers. These results have been summarized by Chance (1); only the necessary details will be reviewed below. The reaction of catalase with hydrogen peroxide has the advantage of involving only one species of a small inorganic molecule reacting with a large heme protein. This reaction is desirable since both the enzyme and substrate have characteristic absorption bands in the visible and ultraviolet. Spectrophotometric recording of the time course of the reactions is therefore possible.

This paper deals with what happens in a biological reaction on a molecular and submolecular level. Evidence for this can be found in the variation of reaction rates with the physical properties as temperature, dielectric constant and coefficient of viscosity.

THEORY

From temperature studies one can find a characteristic heat of activation ΔH_a, provided one is measuring the rate of a single reaction. If it is not controlled by the diffusion rate of the reactants, then ΔH_a may be regarded as a fundamental property of the molecules involved. It has been stated (2) that if a reaction has a heat of activation around 12 Cal/mole, it is probably not diffusion controlled, whereas if ΔH_a is around 3–4 Cal/mole, the reaction is diffusion controlled. The catalase-hydrogen peroxide reaction is interesting since ΔH_a for the overall reaction varies from 1.4 to 2 Cal/mole (3).

If the reaction involves one or more charged molecules, one in general may expect the rate to vary as the dielectric constant (2). One difficulty

[1] Present address: Department of Colloid Science, Free School Lane, Cambridge, England.

here is to distinguish between variations due to changing the composition of the medium from variations due to dielectric constant. We have sought to avoid this difficulty by using two substances which vary the viscosity in the same direction but have opposite effects on the dielectric constant, such as glycerol and methocel.

By varying the viscosity, if one can identify the dielectric effects, one can determine if the reaction is diffusion controlled. To study this one needs to know the variation of the diffusion constant, D, with viscosity. For larger molecules, the diffusion rate probably obeys the Einstein-Smoluchowski relationship, i.e. D is inversely proportional to the coefficient of viscosity, η. For smaller molecules, this relationship cannot be used. Very few values have been published for the variation of diffusion rates

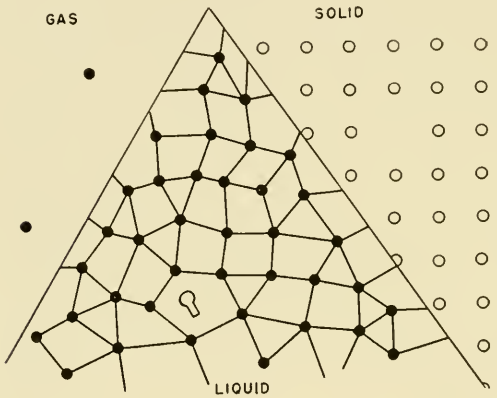


FIG. 1. Solid, liquid and gas molecular configurations. Note the long-range order and lattice hole in the solid, the short-range order and impurity (key) in the liquid, and the complete lack of order in the gas.

with viscosity. This is true, in part, because of the difficulty of finding a simple relationship between D and η. We do not have values for H_2O_2, but have used recent values for the diffusion of O_2 in glycerol and sucrose solutions (4). The diffusion rates of all small molecules in a given solvent are similar (5).

The number of encounters per second between a catalase and a hydrogen peroxide molecule is proportional to the diffusion constant. However, it is naive to conclude from this that all reaction rates are diffusion controlled. The cell theory of liquids developed by many scientists including Leonard-Jones, Popple, Frenkel (5) and Eyring (2) makes it clear that a molecule moves by taking small discrete jumps between quasi-equilibrium positions. Figure 1 contrasts the quasi-equilibrium positions of a liquid with the order of a solid and with the disorder and low density of a gas. In a liquid, if one slows the diffusion rate, one may be slowing the jump frequency. Then one reduces the number of encounters while in-

creasing the length of an encounter. If the encounters are sufficiently long, a reaction will occur for each encounter. Then the reaction rate will vary with the diffusion rate. If, on the other hand, the probability of reaction is lower, so that most encounters do not lead to a reaction, then the reaction rate will depend on the product of the length of encounter times the number of encounters per second. One might hope that one could vary the diffusion rate sufficiently to change from a diffusion independent rate constant to a diffusion controlled constant. As will be seen later, one of the reaction rate constants for our catalase satisfies this condition.

CATALASE-HYDROGEN PEROXIDE REACTION

In discussing the reactions of catalase with hydrogen peroxide we have used Chance's symbols (1). The reaction takes place in two steps:

$$\underset{\text{cat}}{\overset{e-p}{}} + \overset{x}{H_2O_2} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \overset{p}{\text{cat} \cdot H_2O_2}$$

$$\overset{p}{\text{cat} \cdot H_2O_2} + \overset{x}{H_2O_2} \xrightarrow{k_4} \text{cat} + 2H_2O + O_2$$

where the letters above the equations represent the concentrations of the reactants. If x is sufficiently great compared to $e - p$, k_2 may be ignored. Manipulating these algebraically, the concentrations should obey the equations:

$$\frac{dp}{dt} = -p(k_1 + k_4)x + k_1 ex \qquad (1)$$

$$\frac{dx}{dt} = -k_1' ex \qquad (2)$$

where

$$k_1' = \frac{8}{1/k_1 + 1/k_4}.$$

In addition, p will reach a maximum p_1, such that

$$p_1/e = \frac{1}{1 + k_4/k_1} \qquad (3)$$

Reaction 1 may be observed at 405 mμ, since the optical density of p is less than e. Likewise p_1/e may be determined at 405 mμ. On the other hand, reaction 2 may be observed at 230 mμ where H_2O_2 has a stronger absorption than the protein, catalase.

EQUIPMENT

The Beckman DU spectrophotometer, with photomultiplier and adapter, was used in most of our 230 mμ experiments. The amplified output of the photomultiplier is fed through a 60 eps synchronous chopper and then further amplified about five hundred times. This amplified output is displayed both on the oscilloscope and on the Bruel-Kjaer recorder. For these experiments at 230 mμ, a Beckman hydrogen arc was used as the light source; its stability was more than sufficient for most experiments. The temperature in the cuvettes was controlled by coils surrounding the cuvettes. For measurements made at other than room temperatures, a copper constantin thermocouple was inserted next to the cuvettes before and after each reading.

A typical recorded curve is shown in figure 2. The width of the line is due to the recorder pen partially following the sixty cycle signal. The

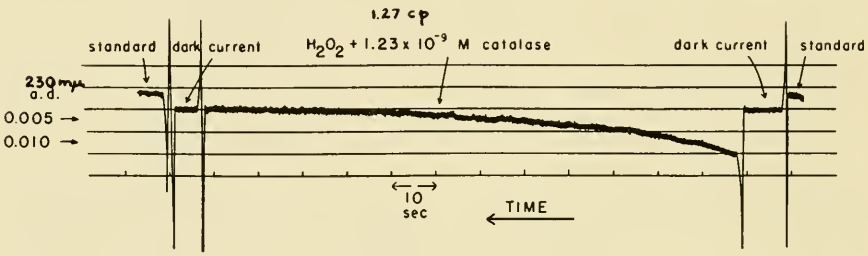


FIG. 2. A typical record at 230 mμ.

stability was checked in each case by recording the dark current level, and the optical density of a solution in a comparison silica cuvette. At the arrow, 0.02 ml of a 0.22 μM solution of catalase was stirred into the H_2O_2 in the cuvette. Experiments showed that the half time, $t_{1/2}$, for this reaction is independent of the exact H_2O_2 concentration, but inversely proportional to the enzyme concentration. In order to obtain meaningful curves it was found necessary to have the enzyme solution at the same viscosity and temperature as the peroxide solution. A difference in viscosity of a few centipoise changed the optical density more than the change due to the hydrogen peroxide disappearing.

The build-up of p to p_1 takes place in a very much shorter period of time. To observe this it is necessary to use a flow system. A thermistor is used as the sensing element to activate a magnetic amplifier which regulates the temperature of the liquid in the control coils. An accessory control system regulates the temperature in the coils surrounding the observation chamber. The temperature of the reactants entering the flow system is monitored continuously with a copper constantin thermocouple.

The optical density in the observation tube could be followed either

with a stopped flow method if the half times were greater than 5 msec or with an accelerated flow method for half-time from 0.2 to 5 msec. In the former case, the reaction curve was recorded on the Bruel-Kjaer apparatus. For accelerated flow determinations, the velocity of flow was displayed on the x axis of the oscilloscope and the optical density on the y axis. A long persistence screen was used; the curves were either photographed with a Land camera, or drawn with wax pencil over the protective grid, measured and cleaned off. In order to vary the hydrogen peroxide concentration over a factor of three hundred, it was necessary to use both stopped flow and accelerated flow measurements, since $t_{1/2}$ varies by a similar factor.

For most of the flow system experiments, the total optical density change during the reaction was about 0.00015 with a 0.1 μM catalase solution. This is smaller by a factor of roughly twenty than the corresponding change using cuvettes since the path length is shorter by this factor. The tungsten lamp was operated from a stabilized power supply similar to one described by Chance (6). The noise level under ideal operating conditions corresponded to an optical density change of 0.00001 or less. Ideal operation meant, among other things, doing experiments only from 1 to 5 A.M. Most of our experiments were made between 12 noon and 12 midnight; since the noise level was appreciably higher at this time, it limited the accuracy.

EXPERIMENTAL RESULTS

In the preliminary set of experiments the reactions were followed only with the flow system at both 405 mμ and 230 mμ. Armour powdered beef liver catalase was used. The viscosity and dielectric constant of the medium was varied with glycerol, sucrose and methylcellulose. All three slowed the formation of p at 405 mμ, increased the ratio of p_1/e, but left the reaction at 230 mμ relatively unchanged. The data at 405 mμ led to the conclusion that k_1 was independent of viscosity or dielectric constant, and that k_4 was also independent of dielectric constant but was diffusion controlled. The data from 230 mμ agreed with results that k_1 and k_4 were independent of the dielectric constant and that k_4 was diffusion controlled. However, these results indicated that k_1 increased as the diffusion rate decreased.

We felt it was necessary to repeat these experiments using a more purified catalase preparation. For all the results shown in the graphs and discussed below, Mann Laboratories crystallized beef liver catalase was used. Here the results showed a consistent systematic difference of about a factor or two in k_1 and k_4 as computed from data at 405 as compared with data at 230 mμ. However, the percentage variation with temperature

and diffusion rate of both k_1 and k_4 was identical within the limits of experimental error for both sets of data. Since the data at 405 mμ were close to the noise limit, their spread was greater. Hence, the rate data presented here are based on the cuvette determination at 230 mμ.

The variation of k_1' with temperatures from 5° to 45°C at pH 7.5 is shown in figure 3. The data at 45°C demanded working quite rapidly since the enzyme starts to denature within 5 minutes at 45°C. The graph of log k_1' against T^{-1} shows a linear relationship within experimental error. The slope of this line shows the value of ΔH_a to be 2 ± 0.2 Cal/mole. This value is close to those obtained by Chance (3).

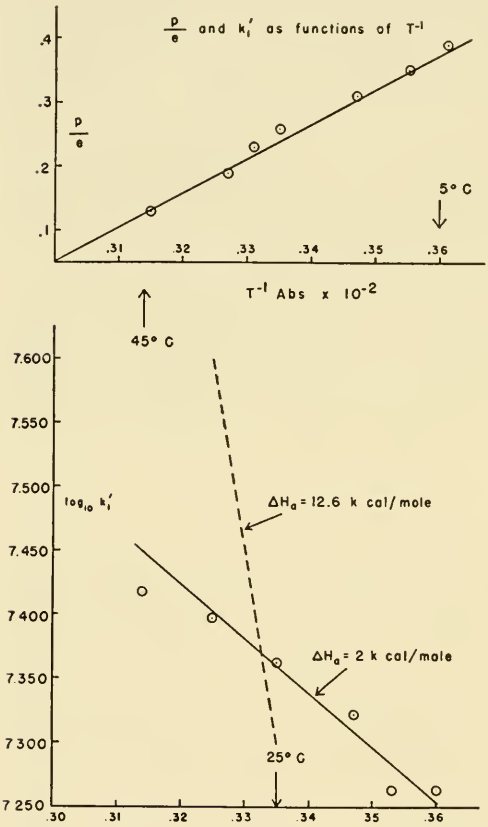


FIG. 3. Variation of k_1' and P_1/e with temperature.

As noted earlier, the constant k_1' is a combination of both k_1 and k_4. To test if the value of ΔH_a was common to both of these constants, we measured p_1/e as a function of temperature in an independent set of experiments. The results are also plotted in figure 5. Here, p_1/e, and not the log, is plotted since the linear scale gave a straighter line. If k_1 and k_4 both had the same temperature dependence, this line should have been parallel to the T^{-1} axis, in contrast to its experimentally observed slope. An identical set of experiments with the flow system gave values for $k_1 + k_4$.

An independent series of measurements was carried out at 25°C at various viscosities of the suspending medium. Chemically pure glycerol was mixed in appropriate proportions with buffer, distilled water and reactants. The results of these measurements are summarized in figure 4. The variation of p_1/e with fluidity (reciprocal of viscosity) is similar to that found

using the powdered catalase. However, with the crystallized catalase k_1' also varied with the fluidity. This variation is similar to that predicted from the 405 mμ results using either the powdered or crystallized catalase.

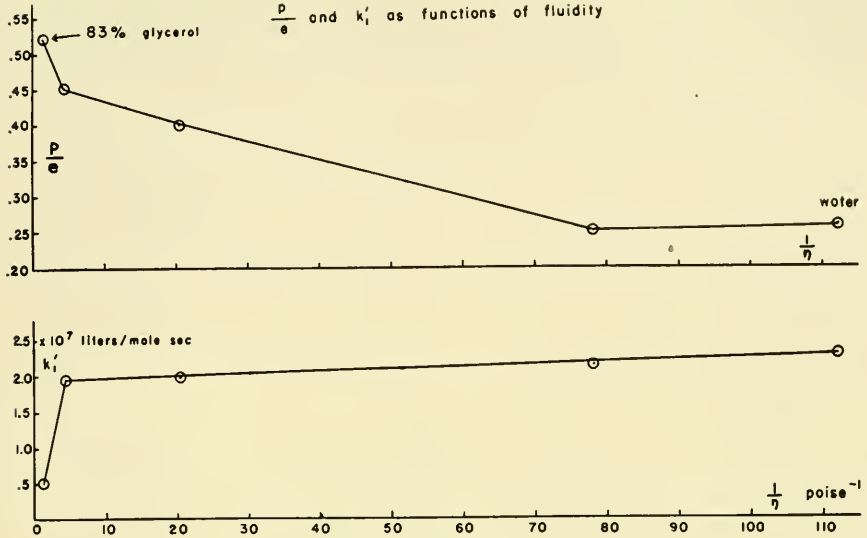


FIG. 4. Variation of k_1' and P_3/e with fluidity.

ANALYSIS AND DISCUSSION

The variations of k_1' and p/e with temperature were used to compute values of k_1 and k_4. These are shown in figure 5. Similar lines, but with a greater spread, can be derived from the data of 405 mμ.

The extreme flatness of the k_1 curve is surprising; this constant is apparently unaltered by temperature changes. Interpreting k_1 on the basis of absolute rate theory, one finds that the free energy of activation is purely an entropy. This entropy must have a large negative value, and may correspond to the reduction in the disorder of the system upon the formation of the intermediate complex, p (7).

The heat of activation for k_4 is greater than that for the fluidity of water. This may be due to differences between the temperature dependence of the fluidity of H_2O and diffusion rate of H_2O_2.

The complex dependence of the diffusion rate constant, D_1, on fluidity, η^{-1}, as illustrated in figure 6, is reproduced from another paper (4). The constant of interest to us here is D_{FICK} for O_2 in glycerol solutions. The initial rise of D as η is raised from that of water is surprising. If k_4 is diffusion dependent, and if the diffusion rate, D, is the same for both O_2 and

H_2O_2, a corresponding increase in k_1' should have been found. However no such rise occurred so we 'smoothed out' this initial rise in D to obtain approximate values for the variation of D for H_2O_2 with η^{-1}. The results of this process for k_1 and k_4 are shown in figure 7. The data support clearly the concept that k_4 is diffusion controlled at 25° for values of η from 0.9 to 65 cp. The scatter of the points could be due, at least in part, to the

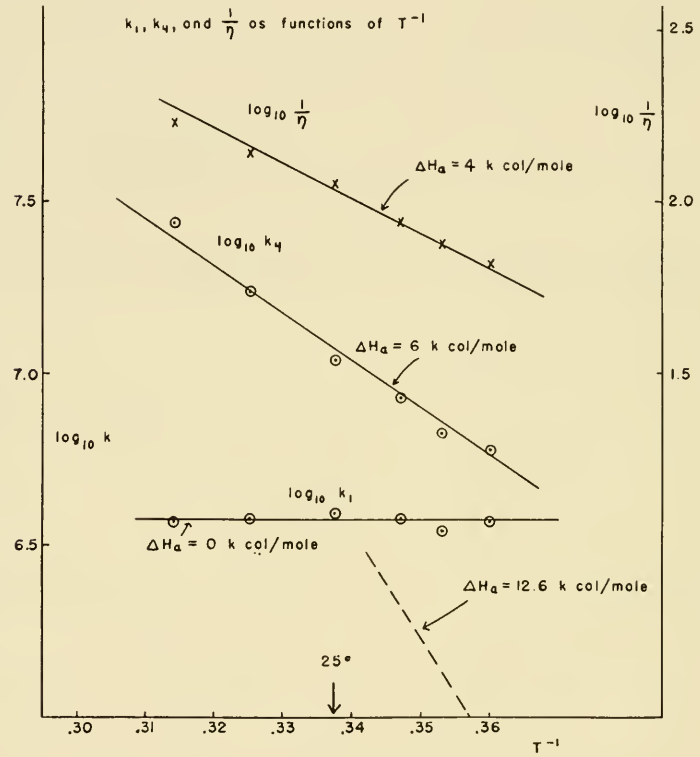


FIG. 5. Variation of k_1 and k_4 with temperature.

differences in D for H_2O_2 and O_2. The values of k_1 are diffusion independent at the low η values, but are diffusion limited at the high values. The same conclusions were reached from the results of the rate of formation of p observed at 405 mμ.

Since k_4 is diffusion controlled, any encounter with a sensitive area on the surface of the molecule must lead to a reaction. In conformity with other usage, we have called this area a reaction cross-section. Since k_1 and k_4 have approximately the same slope in the diffusion controlled region, it is concluded that the sensitive area must have the same size.

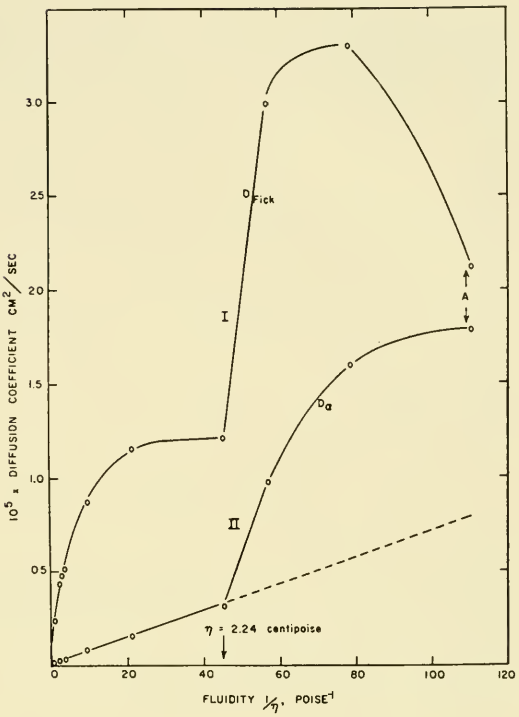


FIG. 6. Variation of D with fluidity (from ref. 4).

The number of encounters of diffusing light molecules of radius r_a with a heavy spherical molecule of radius r_b is given by

$$Z = 10^{-3}\ N4\pi D\ (r_a + r_b)$$

where:

Z is the encounter frequency in liters/mole·sec;
N is Avogadro's number in cgs units;
D is the diffusion rate in cgs units; and
r is in cm.

Using:

$$r_a + r_b \doteq 4 \times 10^{-7}\ \text{cm; and}$$

$$D \doteq 2 \times 10^{-5},$$

one finds that

$$Z \doteq 6 \times 10^{10}\ \frac{\text{liters}}{\text{mole}\cdot\text{sec}}$$

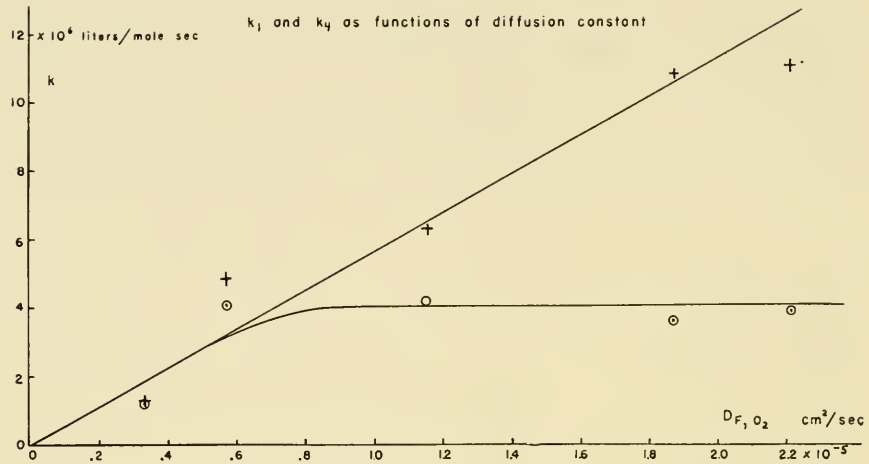


FIG. 7. Variation of k_1 and k_4 with D.

which is far in excess of k_1 and k_4. The approximate surface area of this molecule = 2.5×10^5 A^2. Thus the encounter rate per A^2 is about 2.5×10^5 liters/mole·sec which is far smaller than even k_1. Thus the reaction cross-section, including four hemes, must be of the order of 50–100 A^2. The differences in k_4 could be interpreted as a variation in the reaction cross-section. The fact that these numbers are bigger than 4 A^2 indicates that the reaction must involve a molecular electron orbit which encompasses more than an iron atom.

SUMMARY

The reactions of beef liver catalase with H_2O_2 have been studied under conditions of varying temperature, dielectric constant and viscosity of the suspending medium. These studies have shown that the rate k_1 at which beef liver catalase combines with H_2O_2 to form the intermediate compound is temperature independent from 5° to 45°C, not appreciably altered by the dielectric constant, but dependent on diffusion rates if these are lowered below 0.8×10^{-5} cm^2/sec. The constant k_4, at which the intermediate complex reacts with a second molecule of hydrogen peroxide, is also little altered by variations in the dielectric constant, but is dependent on temperature and diffusion rate. These results are in accord with the prediction of the entropy of activation from absolute rate theory. The results also indicate that any encounter with a portion of the molecular surface appreciably larger than an iron atom will be effective in producing a reaction in the diffusion controlled range.

This investigation was supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council. Portions of the work are included in theses submitted by Berger and Strother to the Graduate School of the Pennsylvania State University in partial fullfillment of the requirements for the Ph.D. degree.

REFERENCES

1. Chance, B. Reaction kinetics of enzyme-substrate compounds, in *Rates and Mechanisms of Reactions*, ed. by S. L. Friess and A. Weisberger. New York: Interscience, 1953, pp. 627–667.
2. Glasstone, S., K. Laidler, and H. Eyring. *The Theory of Rate Processes*. New York: McGraw-Hill, 1941.
3. Chance, B. and D. Herbert. Enzyme-substrate compounds of bacterial catalase and peroxides. *Biochem. J.* 4: 402–414, 1950.
4. Jordan, J., E. Ackerman and Robert L. Berger. Polarographic diffusion coefficients of oxygen defined by activity gradients in viscous media. *J. Am. Chem. Soc.* 78: 2979–2983, 1956.
5. Frenkel, J. *Kinetic Theory of Liquids*. England: Oxford, 1946.
6. Chance, B. Rapid and sensitive spectrophotometry. I. *Rev. Sci. Inst.* 22: 619–627, 1951.
7. Ackerman, E. and R. L. Berger. Entropies of activation of enzyme reactions. In preparation.

METHODS FOR DETERMINATION OF EFFECT OF TEMPERATURE UPON VELOCITY CONSTANTS OF ENZYME REACTIONS

Britton Chance, *Johnson Foundation, University of Pennsylvania, Philadelphia, Pennsylvania*

The bulk of the work on the effect of temperature on enzyme activity has been concerned with the over-all activation energy of the catalyzed reaction and with the loss of enzyme activity caused by the irreversible or the reversible thermal denaturation of the enzyme. While such studies are useful methodologically in determining the range over which preparative procedures or physical and chemical studies of the intact enzyme can be made, they give little data on the nature of the actual activation processes involved in the formation and break-down of the enzyme substrate compounds involved. A summary of such data together with recommendations on the necessity for measuring the effect of temperature upon particular velocity constants involved in the formation and break-down of the enzyme substrate compound was made by Sizer in 1943 (1). The significance of the reversible denaturation in various enzyme-catalyzed reactions or processes, as influenced by heat, pressure and chemical agents, has been discussed by Johnson, Eyring and Polissar (2).

A better understanding of the role of intermediate compounds in enzyme action, together with the development of more sensitive methods for studying enzyme reactions gives, in a few cases, critical data on the effect of temperature on individual reaction velocity constants. Only when the chemical reaction sequence has been specified in some detail can any rigorous, detailed use be made of such thermal data. Thus, this contribution stresses primarily the development of methods suitable for measuring temperature effects on individual velocity constants in enzyme action and gives as examples some of the experimental data that have been obtained.

The classic method for the determination of the effect of temperature upon enzyme reactions has been based upon a study of the overall reaction in which the disappearance of substrate or the formation of product is measured. In only a few enzyme reactions, however, can the overall velocity be attributed to a particular step in the reaction over reasonable ranges of substrate concentrations. Moreover, when temperature is varied over a wide range, as is necessary for accurate determination of reaction heats, the rate determining step may shift from one point in the reaction sequence to another.

In a number of cases, high accuracy of rate data is required because of the small effect of temperature upon the overall rate. Catalase, for example, gives values of 1500 cal. for the bacterial preparation or as low as 600 cal. in the case of the beef liver preparation. Over a 40° temperature range, e.g. between 0° and 40°C, the corresponding change of rate is by a factor of only 1.42 or 1.15, respectively. Highly accurate methods of measurement are obviously needed for these reactions.

Direct measurements of the rate of formation of enzyme substrate compounds are ideal for purposes of determining a single reaction velocity constant and the effect of temperature upon it. Such methods are not always applicable, either because the enzyme-substrate compound is not detectable by physical methods (5) or because the speed of the combination of the enzyme and the substrate is too rapid for kinetic measurements over a reasonable range of substrate concentrations and temperatures. In the case of hemoproteins, the rapid flow method has been applied successfully to the study of the formation of intermediate compounds (6) and Gibson and Roughton (7) have obtained considerable data on the effect of temperature on the kinetics of oxygen and hemoglobin. Only preliminary thermal data are available in the case of catalase and peroxidase due to the extreme rapidity with which the intermediates form and the small amounts of purified material available.

In this paper we present a brief evaluation of three methods for measuring reaction velocity constants in enzyme reactions and the effects of temperature upon them. Applications of the methods will be included and will largely be based upon studies of intermediate compounds of catalase and peroxidase.

DETERMINATION OF REACTION VELOCITY CONSTANTS FROM THE OVERALL REACTION

Catalase and peroxidase kinetics give two examples of the complications involved in determination of reaction velocity constants from the overall reaction. In the case of peroxidase, a relatively straightforward determination of the reaction velocity constants defined by the equation:

$$\begin{aligned} E + S &\underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \\ ES + AH &\xrightarrow{k_4} E + SH + A \end{aligned} \qquad (1)$$

may be obtained, provided that due regard is paid to the limitations of the following equation (8):

$$\frac{dx}{dt} = \frac{e}{\frac{1}{k_4 a} + \frac{1}{k_1 x}} \qquad (2)$$

This equation relates the velocity of disappearance (dx/dt) of hydrogen peroxide to k_1 and k_4. It can be seen that either k_1 or k_4 can be determined for appropriate ratios of the substrate and hydrogen donor concentrations. Conditions for the guaiacol assay of peroxidase activity which permit a determination of these two reaction velocity constants and preliminary values for the effect of temperature upon these two velocity constants are reported (8). However, the range of concentration over which the determination can be made is rather limited, as is the choice of hydrogen donor molecules. This appears to be, however, one of the few cases in which the principal velocity constants, k_1 and k_4, can separately be determined from the overall reaction kinetics and this is because the enzyme-substrate compounds in this reaction have already been studied directly.

In catalase, the effect of temperature upon the velocity constant for the overall reaction is small (fig. 1). A study of the reaction mechanism shows that the rate of disappearance (dx/dt) of substrate depends upon both k_1 and k_4 and dx/dt increases linearly with the concentration of hydrogen peroxide (9, 10) up to 0.5 M (11):

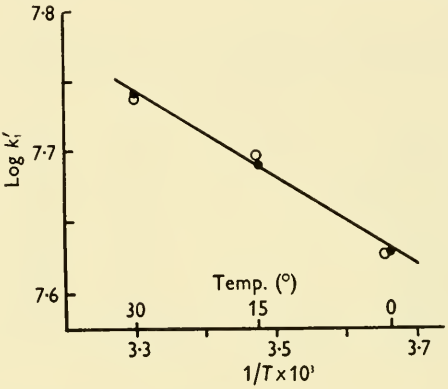


FIG. 1. Effect of temperature upon the overall activity of catalase in the decomposition of hydrogen peroxide. Bacterial catalase activity assayed by the change of ultraviolet absorption due to the disappearance of hydrogen peroxide (3). Data from *Biochem. J.*

$$dx/dt = -xe\left(\frac{2}{1/k_4 + 1/k_1}\right) \qquad (3)$$

Fortunately, from observations of the steady state concentration of the enzyme substrate compound a ratio of the velocity constants can be determined:

$$\frac{p_m}{e} = \frac{1}{1 + \frac{k_4}{k_1}} \qquad (4)$$

and their individual values calculated from the two equations above. Thus, the effect of temperature on the overall reaction kinetics cannot be

attributed to either k_1 or k_4 without direct studies of the effect of temperature upon the intermediate compound. This has been carried out in detail in Beers and Sizer (4) and their values of ΔE and ΔS are given in table 1.

RATE OF FORMATION OF INTERMEDIATES

Gibson and Roughton have studied the effects of temperature upon the rate of formation of intermediates of hemoglobin with its various ligands, and a complete summary of such data is given in figure 2 (7).

In the case of catalase and peroxidase, the rapid flow method has been used to determine the reaction velocity constant for the combination of enzyme and substrate at room temperature. Since the expenditure of enzyme for such studies is rather great, no detailed data are available on the effect of temperature, although in a preliminary study of the effect of temperature upon the reaction of peroxidase and hydrogen peroxide, a very low value has been reported (12). More recently, Ackerman has made a detailed study of the catalase reaction and his results are included elsewhere in the symposium (13).

TABLE 1. EXPERIMENTAL ENERGY, FREE ENERGY AND ENTROPY OF ACTIVATION OF THE TWO REACTIONS, K_1 AND K_4, FOR A MOLAR CONCENTRATION OF HEME AT 25°

Source of catalase	Reaction	$E_{exp.}$ kcal.	ΔF, kcal.	ΔS, e.u.
Horse erythrocyte (4 hemes)	k_1	1.7	7.54	−21.5
	k_4	1.7	6.94	−19.5
Bacterial (4 hemes)	k_1	1.4	7.2	−21.5
	k_4	1.4	6.96	−20.7
Beef liver (3 hemes)	k_1	0.6	7.60	−25.5
	k_4	0.6	7.23	−24.2

Data of Beers and Sizer on k_1 and k_4. Data from *J. Phys. Chem* (4).

A reaction of great physiological and chemical interest is that of oxygen with the reduced cytochrome a_3. This reaction proceeds so rapidly at room temperature that it is necessary to study it at low temperatures in order to obtain measurements over a reasonable range of oxygen concentrations. For isolated particles, a value of 10^7 M^{-1} sec.$^{-1}$ has been obtained at 3°. Preliminary data (14) on the effect of low temperatures on this interesting reaction show that an intermediate between cytochrome a_3 and oxygen becomes rate limiting as the temperature is lowered to −10°.

STEADY-STATE METHOD

It has recently been possible to devise an equation from which the velocity constant for the combination of enzyme and substrate can be computed directly from the kinetics of the intermediate compound (16) without the necessity for measuring very short times required by the procedure described above. The measurement depends only upon the concentration of the enzyme substrate compound in the steady state and the 'half-life'

of the enzyme substrate compound. Both these quantities can be measured accurately and for smaller ratios of a_0 and x_0 and low enzyme concentrations; the half-life of the enzyme substrate compound is long enough to be measured by very simple methods; no flow apparatus being required.

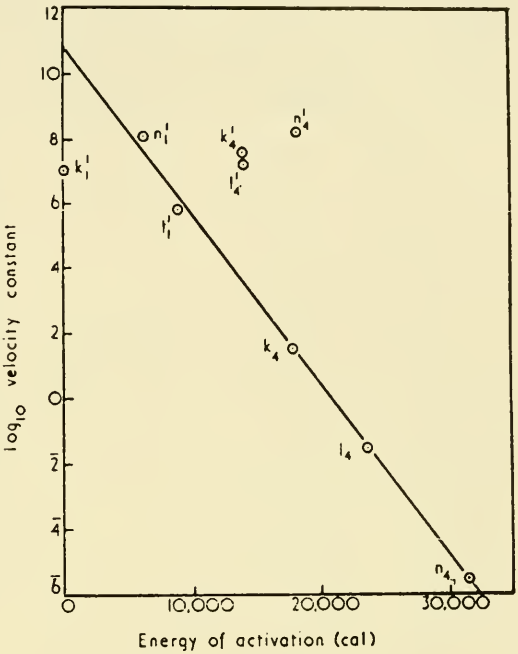


FIG. 2. Gibson and Roughton's thermal data on reactions of hemoglobin with various ligands (7). In the terminology of Gibson and Roughton, the primed values refer to 'on' velocity constants, the unprimed to 'off' velocity constants. The subscripts refer to the binding of the first, second, third and fourth molecule of the particular ligand and the letters k, l, and n refer respectively to O_2, CO, and NO molecules. Data from Faraday Society.

The equation, which has been derived elsewhere (16), is as follows:

$$k_1 = \frac{1}{(e - p_m)\, t_{\frac{1}{2}off}} \qquad (5)$$

where e is equal to the initial concentration of the enzyme, p_m is maximum concentration of the intermediate compound under particular experimental conditions (this is also the steady state concentration) and $t_{\frac{1}{2}off}$ is the time interval from the formation of the enzyme substrate compound until its concentration has fallen to half its maximum value. Figure 3 serves to illustrate the measurement of these quantities.

It should be pointed out that these measurements refer to the rate-limiting intermediate in the reaction sequence. Fortunately, this intermediate has the largest steady-state concentration and is most readily detected by physical methods in the case of peroxidase and catalase. In this case, *equation 5* applies to the rate of combination of enzyme and

substrate. Otherwise, a velocity constant further along the reaction sequence may be measured (16).

In addition to the advantage that a rapid flow apparatus is not needed, this equation has the advantage that neither substrate nor hydrogen donor concentrations need be known and in cases where e is measured independently, for example, by an activity assay or by physical methods, the experiment need only give the fractional amount of enzyme in the form of enzyme substrate and compound and the half-life of the intermediate. Both these quantities can be measured rather accurately.

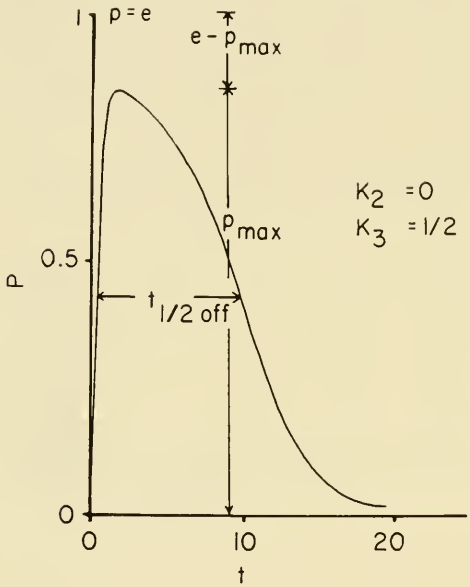


Fig. 3. Illustrating the quantities measured from the kinetics of the enzyme substrate compounds in order to compute the velocity constant for the formation of the intermediate. The diagram is a differential analyzer solution for the kinetics of appearance and disappearance of the enzyme substrate compound of *equations 1* and *2* for the conditions 1 μM substrate. The units of the abscissa are seconds for k_1 is equal to 10^6 M^{-1} sec^{-1}. The value of K_3 ($= k_4 a$ of *equations 2* and *3*) is 0.5 sec^{-1} and k_2 of *equation 1* is 0. The figure illustrates the measurement of the quantities $e - p_m$ and $t_{\frac{1}{2}off}$ of *equation 5*. For more details see ref. 16. Data from *Arch. Biochem.*

This equation has been applied to the determination of the reaction velocity constant for the formation of the intermediate compound in peroxidase action and the effect of temperature upon this velocity constant. These data are illustrated in figure 4 and it is seen that a heat of 3600 cal. is obtained. The room temperature value for the velocity constant is in close agreement with that obtained directly by the rapid flow method. In cases where the substrate (x_0) and hydrogen donor concentrations (a_0) are known, the following equation may be used to calculate k_4, the velocity constant for the reaction of the enzyme-substrate compound and hydrogen donor:

$$k_4 = x_0/a_0\, p_m t_{\frac{1}{2}off} \qquad (6)$$

and figure 4 includes the effect of temperature upon this velocity constant.

Application of This Equation to the Kinetics of Cytochrome a_3 and Oxygen. *Equation 5* above has a sound theoretical basis for the peroxidase-type kinetics, and it is very probable that it is an approximation to

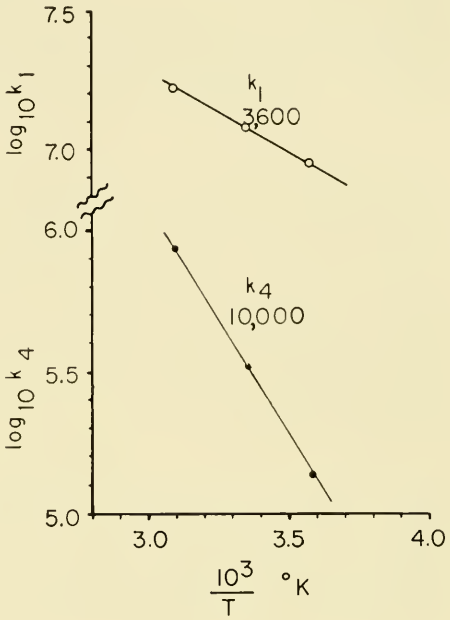


Fig. 4. Effect of temperature on the reaction velocity constants k_1 and k_4 as determined according to *equations 5* and *6* (from experimental data similar to that in fig. 3). The heats of activation are indicated for the two velocity constants. (The limitations to the interpretation of these data are described in the text, expt. 630a.) For more details see ref. 16. Data from *Arch. Biochem.*

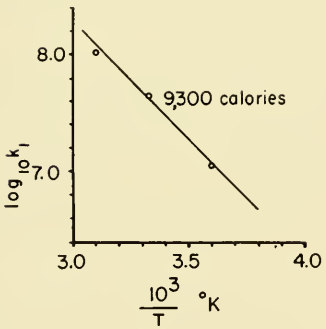


Fig. 5. A plot of the values of k_1 computed for the reaction of oxygen with cytochrome a_3 of baker's yeast cells according to *equation 5*. The large value of heat of activation suggests the presence of intermediate compounds between oxygen and cytochrome a_3 (expt. 630b). For more details see ref. 16.

the value of k_1 for the more complex cytochrome type kinetics, especially when the cycle of oxidation and reduction of the cytochrome has a reasonable 'plateau.' Experimentally, it is easy to apply this equation to cytochrome kinetics, and some very preliminary results are included in figure 5. The value of k_1 which has been determined in these conditions agrees rather well with preliminary values for k_1 obtained in the rapid flow

apparatus (14, 15), although the values are lower than the estimate of Ludwig and Kuby (17). Temperature variation has a surprising large effect upon the value of k_1 and it is not at all certain, due to the limitations in the validity of *equation 5*, that this temperature variation can be truly attributed to the combination of oxygen with cytochrome a_3, since other data have already pointed to the existence of intermediates between cytochrome a_3 and oxygen. Thus this large heat of activation could be attributed to such steps.

SUMMARY

Three approaches to the problem of the determination of the effect of temperature upon the speed of enzymatic reactions have been briefly outlined. Of these three, the direct determination of these velocity constants by means of the rapid flow method is by far the best, but, by means of a simple equation, based on the steady state reaction kinetics of the intermediate compound, many advantages of the direct method have been retained without the need for the measurement of very short times by the rapid flow apparatus.

REFERENCES

1. Sizer, I. W. *Advances Enzymol.* 3: 35, 1943.
2. Johnson, F. H., H. Eyring and M. J. Polissar. *The Kinetic Basis of Molecular Biology.* New York: Wiley, 1954.
3. Chance, B. and D. Herbert. *Biochem. J.* 46: 402, 1950.
4. Beers, R. F. and I. W. Sizer. *J. Phys. Chem.* 57: 290, 1953.
5. Chance, B. and R. R. Fergusson. In: McElroy and Glass, *The Mechanism of Enzyme Action.* Baltimore: Johns Hopkins Press, 1954.
6. Chance, B. *Advances Enzymol.* 12: 153, 1951.
7. Gibson, Q. H. and F. J. W. Roughton. Faraday Society Discussion: *The Physical Chemistry of Enzymes.* Aberdeen: Univ. Press, 1955, p. 203.
8. Chance, B. and A. C. Maehly. In: Colowick and Kaplan, *Methods of Enzymology.* New York: Acad. Press, 1955, vol. 2, p. 772.
9. Chance, B., D. S. Greenstein and F. J. W. Roughton. *Arch. Biochem.* 37: 301, 1952.
10. Chance, B., D. S. Greenstein, J. Higgins and C. C. Yang. *Arch. Biochem.* 37: 322, 1952.
11. Ogura, Y. *Arch. Biochem.* 57: 288, 1955.
12. Bonnichson, R. K., B. Chance and H. Theorell. Acta chem. scandinav. 1: 699, 1947.
13. Ackerman, E. *Influence of Temperature on Biological Systems.* Washington: Am. Physiological Soc., 1957.
14. Chance, B., B. Mackler, W. Holmes and J. Higgins. Abstracts and Papers, American Chemical Society Meeting, Dallas, Texas, 1956, p. 120.
15. Chance, B. Faraday Society Discussion: *The Physical Chemistry of Enzymes.* Aberdeen: Univ. Press, 1955, p. 208.
16. Chance, B. *Arch. Biochem.* To be published.
17. Ludwig, G. D. and S. A. Kuby. *Fed. Proc.* 14: 257, 1954.

TEMPERATURE DEPENDENCE OF CHOLINESTERASE ACTIVITY

Leigh E. Chadwick, *Physiology Division, Chemical Warfare Laboratories, Army Chemical Center, Maryland*

The hydrolysis of acetylcholine (ACh) by cholinesterases (ChE's), because of its recognized physiological importance, has been investigated intensively for 30 years. Recently, an added stimulus has been the fact that certain insecticides and chemical warfare agents owe their potency to interference with this reaction; but, considering the volume of study devoted to the system, some of its fundamental properties have received rather cursory attention. Among them is its temperature dependence, a subject with numerous theoretical and practical implications, the latter especially among poikilothemic organisms. Though work of the past few years has added to our knowledge of this phase, there are still many unanswered questions.

Existing information may be summarized conveniently under three main headings. *1*) temperature and rate of hydrolysis, *2*) inactivation of ChE by heat, and *3*) effect of temperature on rate of reaction with inhibitors. Since data on all three aspects are obtained from study of hydrolysis rates, there is inevitably some overlap in this classification, as will be apparent in the discussion below.

TEMPERATURE AND RATE OF HYDROLYSIS

The ability of ChE's to split ACh and some other esters has been examined as a function of temperature with enzymes from: *mammalian blood* (2, 8, 12–15, 17, 18, 21, 26, 34, 39, 40, 45, 46); *ox cortex and caudate nucleus* (35); *intestine of the fish, Ameiurus* (29); *developing eggs of the grasshopper* (44); *arthropod nerve cord*: *lobster* (7), *cockroach* (37); *fly heads*: *Dacus* (38), *Musca* (10). In addition, it is possible that the enzyme from developing eggs of *Melanoplus*, investigated by Carlson (9) with methyl butyrate as substrate, is the same as the ChE of Tahmisian (44), since the method of preparation and the temperature response were very similar. Smallman and Wolfe (43) have produced data, still unpublished, that supplement those cited above for the head of the house fly.

Despite the number and variety of references, the exact nature of the relationship between temperature and ChE activity is still in doubt. Some of the reports deal with specific, others with nonspecific esterases, and in many the temperature data are only incidental. There seem also to be

differences in the temperature response of enzymes of the same type from different animals, but so far this question has been very imperfectly surveyed.

Moreover, most published records do not include a measure of the experimental variability, so that one is denied the opportunity of using statistical methods for narrowing the choice among the various types of relationship that could be fitted by eye to the results, and one is often left to wonder also whether apparent differences between enzymes from different sources, or in the same enzyme in the hands of different workers, are real or fortuitous.

A more fundamental difficulty is posed first by the nature of the reaction, which is thought to have the form

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} ES^* \rightarrow E + \text{products} \qquad (1)$$

in which ES* is the 'activated complex' of the theory of absolute reaction rates (20); and secondly by the limitations of present techniques for determining reaction velocity, which measure only the rate of disappearance of substrate or the rate of accumulation of products. Thus the data obtained refer only to the speed of the overall reaction, a limitation by no means peculiar to the hydrolysis of ACh by ChE. The formal equivalent of the equilibrium constant, K, is, therefore, provided one may assume a steady state,

$$K = k_1/(k_2 + k_3). \qquad (2)$$

Evidently, plotting log K in the usual manner against $1/T_{abs}$ gives a curve whose slope is a composite function that includes, in undetermined proportions, both heat of reaction to form ES and heat of activation to convert ES to ES*. For this reason, measurements of the temperature dependence of K cannot be used to derive meaningful values for the standard thermodynamic constants, except under the limiting conditions, $k_2 >> k_3$, $k_2 << k_3$ (cf. ref. 25; pp. 176–177). Some authors have adopted such premises in order to calculate the constants in question, though without direct justification, which would require a means of measuring the relative magnitude of k_2 and k_3.

However, the reaction velocity is

$$v = k_3 \cdot E_0 \cdot K \cdot S/(1 + K \cdot S) \qquad (3)$$

where E_0 is the total concentration of enzyme and S the concentration of substrate. Thus, at high substrate concentrations,

$$V_{max} = k_3 \cdot E_0. \qquad (4)$$

The temperature dependence of the step, $ES \xrightarrow{k_3} ES^*$, may therefore be evaluated by computing V_{max} in the usual way from the pS-activity curves obtained at different temperatures. If the plot of log V_{max} against $1/T_{abs}$ is linear, one may then estimate the apparent energy of activation, ΔE^*, from the Arrhenius equation; and the true energy of activation, ΔH^*, as $\Delta E^* - RT$. The standard free energy and entropy changes, ΔF^* and ΔS^*, can also be calculated if the molar concentration of enzyme is known, but this information is not usually available for ChE preparations.

A prime obstacle to the application of this method of analysis is the fact that most sets of data for ChE's do not give straight lines in the Arrhenius and similar formulations. Our results with homogenates of house fly heads, which contain a specific ChE, are reasonably typical (fig. 1).

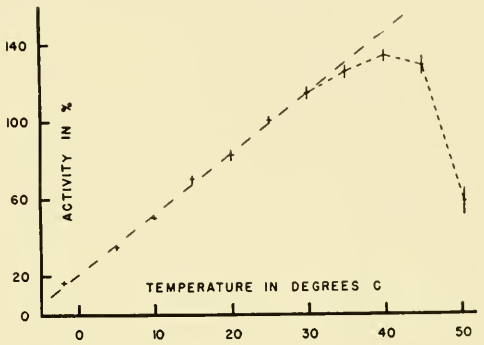


FIG. 1. Activity of fly head ChE as a function of temperature. Vertical bars show the range of variation for ±3 S.E. The straight dashed line was calculated by the method of least squares to fit the data at 30°C and below. Data from (10).

Here the activity measured was directly proportional to temperature, up to 30°C (10). Within the errors of measurement, similar relationships of nearly the same slope have been found with horse serum (26, 21, 15), for human serum and erythrocytes, respectively (40), with heads of the oriental fruit fly (38), with nerve cords of the cockroach (37), and with the distal portion of the lobster nerve cord (7). For the proximal portion of the lobster cord, the slope was somewhat steeper. The measurements of Tahmisian (44) with the ChE of developing grasshopper eggs do not define the shape of the curve below 35°C, but Carlson (9), using methyl butyrate as substrate with a very similar preparation, found a nearly direct ratio between activity and temperature over the range from about zero to 45°C. A relationship of this form is evidently characteristic of many crude ChE preparations, and shows that the apparent energy of activation, whatever significance may be attached to it, is often not constant but temperature-dependent. Thus, the indication is that the true temperature relationships of the reaction are being obscured by compli-

cating factors, although we have as yet little notion of what these factors may be.

In some instances, data that seem to fit the Arrhenius formulation have been secured. Ormerod (34), studying the hydrolysis of eight derivatives of benzoylcholine by horse serum ChE, obtained apparent energies of activation ranging, with the different substrates, from 5.1 to 13.3 Cal/mole. Davies (15) found a constant ΔE^* of 4.2 to 4.6 Cal/mole for hydrolysis of ACh by partly purified preparations of this same enzyme; but with whole or diluted serum he found that the energy requirement decreased with increase in temperature (fig. 2).

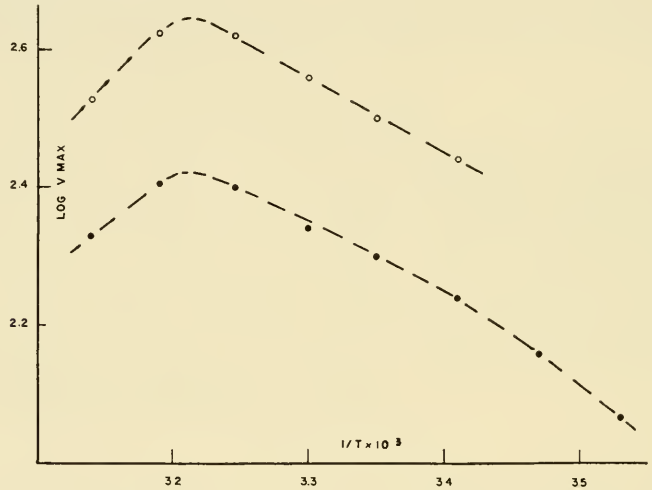


Fig. 2. Activity of horse serum ChE as a function of temperature. *Open circles:* dialyzed preparation; *solid circles*, 20% serum, not dialyzed. Data from (15).

Irrespective of the exact form of the relationship, the increment in ChE activity with rise in temperature is relatively small. The Q_{10} in the physiological range is usually of the order of 1.3–1.5 and falls as temperature is increased. Average values of about 1.7 reported by Abdon and Uvnäs (2) for the plasma enzyme of two human subjects are exceptionally high. The nature of the activity-temperature relationship will be reconsidered below after the data on heat inactivation of the enzyme have been reviewed.

As is well known, the pS-activity curve for hydrolysis of ACh by the so-called true or specific ChE's shows a definite maximum, usually at substrate concentrations of about 0.01 M. The decline in activity at higher substrate concentrations is attributed to formation of an inactive complex between one molecule of enzyme and two of ACh:

$$ES + S \underset{k_5}{\overset{k_4}{\rightleftharpoons}} ESS \qquad (5)$$

The thermal dependence of this reaction was investigated for the ChE of human erythrocytes by Shukuya (40), who found that the equilibrium constant was independent of temperature, so that the standard enthalpy change appeared to be nil. The change in standard free energy was calculated as −2.7 Cal/mole at 310.5°K, and the standard entropy change as +8.8 cal/mole/degree.

Reaction 5 does not occur with the nonspecific ChE's, to which type the serum enzymes belong in most mammals that have been studied; and its temperature dependence has not been investigated with the specific ChE's of nonmammalian organisms.

INACTIVATION OF ChE BY HEAT

At temperatures slightly above the physiological range, evidence of gradual inactivation of ChE is ordinarily seen, and permanent destruction

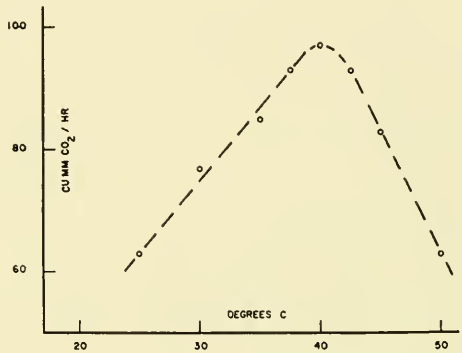


Fig. 3. Activity of horse serum ChE as a function of temperature, to show maximum at about 40°C. The decline at higher temperatures is largely reversible, for short exposures. Data from (21).

of enzyme, though varying to some extent with the source and other conditions, commonly is extensive with exposures of more than a few minutes to temperatures of 55°C or higher (1, 4–6, 10, 13, 15, 16, 19, 21, 23, 26, 30, 31, 36, 38, 44). Many of these references give only incidental or qualitative information.

According to Glick (21) and Davies (15), maximal activity of horse serum ChE occurs at 40°C (fig. 3), although there is little permanent inactivation at temperatures below 50°C. Part of the decline in activity at temperatures above 40°C must therefore be reversible. Irreversible denaturation requires about 60 Cal/mole (23, 15), and has a biphasic course, being rapid initially and slower thereafter; both phases follow first-order kinetics (15). Reversible inhibitors, such as prostigmine and methylene blue, afford some protection (23), but previously denatured serum does not (15), while destruction is accelerated at extreme values of pH and in solutions of low ionic strength (15). The energy requirement for denaturation of the specific ChE of ox erythrocytes is similar, about 61 Cal/mole (13).

The specific ChE of fly heads is more sensitive since some irreversible loss of activity occurs with short exposures to temperatures no higher than 35°C (fig. 4). However, maximal activity is again found at about 40°C, in the usual 20- to 30-minute measurement (fig. 1). Denaturation requires some 44 Cal/mole and is biphasic, with each phase following first-order kinetics as with the serum enzyme. But with fly head ChE there is no convincing evidence of any reversible inactivation by heat, for the undenatured fraction maintains a direct proportionality between activity and temperature up to 50°C or higher (10).

The response of serum ChE's at temperatures above 35°C agrees, at

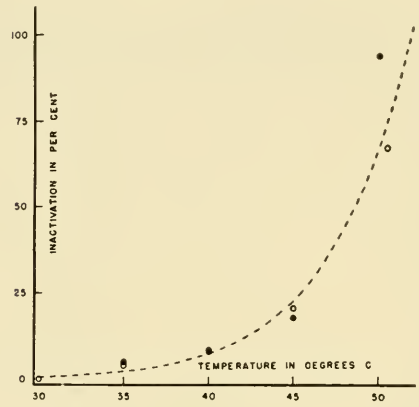


FIG. 4. Denaturation of fly head ChE by heat. *Open circles* computed from data of fig. 1, on assumption that the activity of undenatured enzyme is directly proportional to temperature throughout the range of measurement. *Solid circles* determined by incubating tissue samples at the indicated temperatures for 20 minutes before returning them to 25°C for addition of substrate and assay. The curve shown was fitted to the data by the method of least squares, in the Arrhenius transformation, and corresponds to an apparent energy of activation of 44,550 cal/mole. Data from (10).

least qualitatively, with numerous examples reviewed by Johnson *et al.* (25), in which the apparent energy of activation changes with temperature as the result of a shifting equilibrium between active and reversibly denatured forms of the enzyme. Alternatively, one might invoke the suggestion of Kavanau (27), that some enzymes develop an optimal configuration for combining with substrate only over a rather narrow intermediate range of temperatures. However, neither model accounts satisfactorily for the observations with fly head ChE; for here there seems to be no reversible inactivation, while it is hardly reasonable to suppose the enzyme is still unfolding toward an optimal configuration at 50°C, a temperature at which permanent inactivation occurs in a few minutes and one which is several degrees above the thermal death point for the species.

Nevertheless, the theory of absolute reaction rates is sufficiently comprehensive to permit alternative explanations (25). Even if one discounts

the likelihood that unidentified inhibitory components of the crude preparations obscure the true temperature relationships of the enzyme-substrate reaction, there remains the chance that the heats and entropies of activation of the several steps represented by k_1, k_2 and k_3 of *reaction 1* might be so balanced as to produce a direct proportionality between hydrolysis rate and temperature over a 50° range, although no fully comparable example seems to have been reported. Both possibilities are of course almost wholly speculative in the present state of our knowledge of the system; and experimentally only an attempt to establish or eliminate a role of inhibitory impurities would seem to be feasible within the limitations of current techniques.

At the same time, one may wonder whether the temperature response of fly head ChE and other preparations with similar properties might not be explained more simply by supposing that the rate limiting process in not one that involves the thermodynamic aspects of the enzyme-substrate reaction, but rather some other type of energy barrier whose height is inversely proportional to temperature. Such a suggestion is by no means new. Galehr and Plattner (17) concluded, on the basis of their measurements of Q_{10} values of only 1.3 for human blood ChE, that a physical process was concerned; and they proposed that diffusion of ACh to active sites on the enzyme was the limiting factor. More than the low temperature coefficient, which they stressed, the direct ratio between activity and temperature favors the notion that the rate might be regulated by diffusion. However, attempts to test this hypothesis by altering the viscosity of the medium were made by Kodera (28), with inconclusive results. We too have reported that, although the rate of hydrolysis is slowed in viscous solutions, it is not directly proportional to viscosity in tests made in solutions of sucrose or glycerol (11); but of course we do not know what other complications the addition of these substances in high concentrations may have introduced. Still, acceptable evidence that diffusion is the limiting factor has not been provided; and one must remember too that Davies' (15) data with partly purified preparations (fig. 2) and possibly those of Ormerod (34) with benzoylcholine derivatives fit an exponential formulation, even though most other observations do not.

Thus it seems that no present hypothesis accounts satisfactorily for all the observations, although various explanations are conceivable within the framework of existing theory. Only further comparative work will resolve the question. The problem is both basically important and common to all the ChE's, from whatever source, so that a solution of the dilemma with any one of these enzymes would shed light on the properties of all of them, and perhaps upon unrelated systems as well.

EFFECT OF TEMPERATURE ON RATE OF REACTION OF ChE WITH INHIBITORS

A third phase of this discussion concerns the manner in which temperature influences the effect of certain ChE inhibitors. Here the available information is even more spotty than in the areas already considered. Differing types of temperature response have been observed, depending apparently on the nature of the inhibitory mechanism as well as on the source of enzyme; and with some inhibitors, the mechanism may vary with the inhibitor concentration.

Shukuya (41) found that urethane at about 0.2 M concentration inhibited human serum ChE reversibly at temperatures between 20° and 37.5°C, with some reduction in degree of inhibition as temperature increased. With 0.8 M urethane, however, inhibition at temperatures of 35°C and above was irreversible, and had a positive temperature coefficient. Here the apparent energy of activation was about 65 Cal/mole, from which fact protein denaturation was inferred. Similar results were obtained with Na-salicylate (42), with which reversible inhibition, little affected by temperature, was observed at an inhibitor concentration of 0.15 M. With 0.27 M Na-salicylate, enzyme was destroyed, and the process was temperature dependent, with an apparent energy requirement of about 42 Cal/mole.

Goldstein and Doherty (24) had earlier noticed a difference in the mode of inactivation of ChE by $HgCl_2$ at low and high concentrations. The temperature coefficient for inactivation at high concentrations was large. Prostigmine gave no protection, but BAL (dimercaptopropanol) did. However, BAL failed to reverse such inhibition as had already occurred. Although H_2S alone was not inactivating, H_2S in the presence of $HgCl_2$ enhanced the degree of inhibition; and this increase in inhibition was shown not to be due to formation of HgS.

In these instances, there is little doubt that irreversible inactivation with high concentrations of inhibitor resulted from denaturation of the enzyme protein, even though the attempt by Goldstein and Doherty (24) to show that disruption of sulfhydryl linkages was the mechanism gave somewhat contradictory results.

Goldstein and Doherty (24) stated further that the temperature coefficient for inactivation by $HgCl_2$ was surprisingly high in comparison with the coefficient for physostigmine determined in their earlier work; however, I have been unable to find pertinent data in the reference (22) they cite. There is nevertheless little doubt that their statement is correct, for physostigmine acts as a competitive inhibitor and low temperature coefficients have been observed with other inhibitors of this type.

Thus, for example, Nachmansohn *et al.* (32) gave as a rough estimate Q_{10} of about 2 for the inactivation of electric eel ChE by DFP (di-isopropylfluorophosphate) between temperatures of 7° and 22.5°C. The same

authors later (33) reaffirmed this value, with observation at 10° and 23°C; but my calculations from their reported results indicate a Q_{10} between 1.22 and 1.44, i.e. somewhat less than their own estimate and much like that usually found for hydrolysis of ACh by ChE's.

More detailed measurements of the effect of temperature on the inhibition of ChE by DFP were made by Chadwick and Lovell (10) with fly heads. They found that the specific reaction rate, of the order of 10^6 liters/mole DFP/minute at 25°C, was almost directly proportional to temperature between 15° and 35°C. (fig. 5). The average Q_{10} for the inhibitory

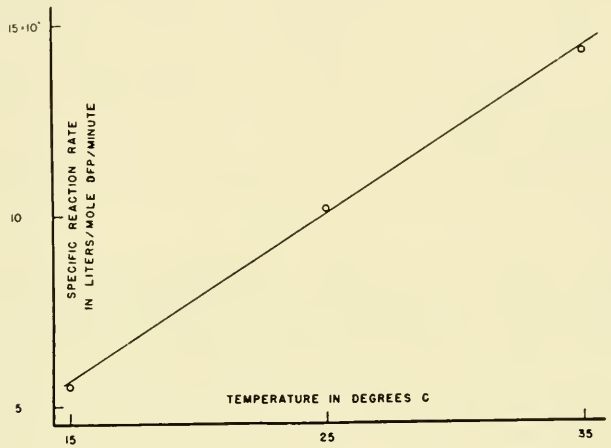


FIG. 5. Rate of reaction of fly head ChE with DFP as a function of temperature. *Open circles:* average reaction rates measured 4 minutes after mixing of enzyme and inhibitor. The straight line shown was fitted to the data by eye. Data from (10).

reaction was 1.58 as compared with 1.34 for the hydrolysis of ACh by this enzyme over the same temperature range.

Aldridge (3) studied the inhibition of rabbit erythrocyte ChE by compound E-600 (diethyl *p*-nitrophenyl phosphate, also known as paraoxon). In contrast to our results with DFP, his data, at temperatures of 19° to 37°C, are in reasonable agreement with the Arrhenius formulation, from which he calculated an apparent energy of activation of 10.6 Cal/mole. Aldridge also determined the temperature dependence of the nonenzymic hydrolysis of E-600 and its dimethyl analog, and found apparent energies of activation of 18.5 and 16 Cal/mole, respectively. ChE inhibited by the dimethyl compound regains activity relatively rapidly after removal of excess inhibitor; and Aldridge showed that this recovery process is temperature-dependent, with an apparent energy requirement of 14.4 Cal/mole. On the basis of these results he states (p. 446) that "it is clear that the in-

hibitory reaction is a chemical reaction and not a simple adsorption". This conclusion is in accord with much other evidence.

The reversible, competitive inhibition of ACh hydrolysis by choline was studied by Davies (15) with horse serum ChE, at temperatures between 15° and 45°C. Davies concluded that the activation energy for formation of the enzyme-inhibitor complex was about −9.8 Cal/mole below 35°C, with an associated entropy change of about −20 E.U., and about −54 Cal/mole above 35°C, with the corresponding change in entropy, −163 E.U. There appears to be no formal objection to these calculations; however, the values obtained depend upon an estimate of the temperature dependence of the equilibrium constant, K_I, for the reaction between enzyme and inhibitor; K_I depends in turn upon the quotient of estimates for the equilibrium constants, K_S and K_S', of the reaction with substrate in the presence and absence of inhibitor. The estimates of K_I are therefore quite sensitive to experimental error in the determination of K_S and K_S'; recalculation of these constants from Davies' data, fitted by the method of least squares to the Lineweaver-Burk formulation, suggests that there is too much uncertainty in the computed values of K_I to warrant placing much reliance on figures Davies reports for the various thermodynamic quantities. With about the same degree of experimental variability, Shukuya (40) concluded that there was no significant change with temperature in the value of K_S. In studies with red cell ChE, Butterworth (8), cited by Davies (15), found that K_I for choline inhibition decreased with increasing temperature, in contrast with Davies' own results with the serum enzyme. The safest conclusion at present would seem to be that we do not yet have sufficiently good data for the values of the equilibrium constants at different temperatures to permit us to state with confidence what form their temperature dependence may take. This means that we are also unable at this time to reach any quantitative conclusions with regard to the temperature dependence of the reaction between ChE and any competitive reversible inhibitors. This limitation does not apply to situations where the inhibition, though competitive, is virtually irreversible, as it is with DFP and many other organic phosphates, such as those studied by Aldridge (3).

Davies (15) also attempted to measure the temperature dependence of the reaction between horse serum ChE and eserine, but was unable to obtain data that satisfied the criteria for reversible competitive inhibition. Since the competitive nature of this reaction was already well established, he concluded that there must be some undetected defect in the experiments.

Roan and Maeda (38) report the degree of inhibition of *Dacus* ChE with 8 inhibitors, principally organic phosphates, at three different temperatures; but their data are unfortunately not given in a form suitable for calculation of the reaction rates, and hence cannot be applied to the problem here under consideration.

To derive useful generalizations from these scattered observations concerning the effect of temperature on ChE inhibition is difficult. We are clearly confronted with processes of several different types, and for none of them do we have really adequate information. Certain non-competitive inhibitors are evidently denaturants at high concentrations. Apparently they act in some different, though wholly unexplained, manner when present in lower concentration. Parallels for this situation have been discussed by Johnson *et al.* (25). The competitive inhibitors, on the other hand, give results that, in their general form and in their variety, point to the same complexities that are seen in the reaction of the enzyme with substrates. This was perhaps to be expected, since competitive inhibitors are presumably combining with the protein in the same manner and at the same sites as the substrate. One judges that considerably more work must be done with a greater variety of inhibitors and ChE's before we shall be able to draw valid general conclusions about the effect of temperature on these various inhibitory processes.

CONCLUSION

This review reveals first of all how incomplete our information is in all three of the areas considered. As yet, experiments on the temperature dependence of ChE activity have not covered enough ground to contribute greatly to our understanding of the nature of this group of enzymes and of their reactions with substrates and inhibitors. Partial though our knowledge still is, it is nevertheless sufficient to throw into relief the fact that the reactions concerned in the three types of process discussed are in each case less simple than is generally taken for granted. Though it was by varying the temperature at which the experiments were done that these complexities were brought into the foreground, this does not imply that the complicating factors are absent or negligible when temperature is kept constant, although that is the tacit assumption of many workers. Thus the temperature data may even now serve the useful purpose of cautioning one against adopting an oversimplified view of the preparations that are ordinarily used in kinetic studies with ChE's.

Superficially regarded, these disturbing factors may seem merely an annoying obstacle to our understanding of the system, or a distraction tending to draw our attention away from the primary goal of our enquiry; but they should more properly be welcomed as affording added opportunities for probing the heart of the problem. We shall not explain these anomalies without in the process learning something significant about ChE itself.

The bibliography to follow does not contain one single reference in which the temperature response of ChE has been studied in a living organism. This is a deplorable admission from an entomologist who has himself done

some work with ChE and its inhibitors. However, the problem is not one of interest only to entomologists; for surely we all have something to learn by attempting to extend our temperature studies of this vitally significant system to truly vital situations.

SUMMARY

The literature on the response of cholinesterases (ChE's) to temperature change has been reviewed. The available data are discussed under three headings: *1*) temperature and rate of hydrolysis, *2*) inactivation of ChE by heat and *3*) effect of temperature on the rate of reaction with inhibitors.

The temperature coefficient of ChE-catalzyed hydrolysis of acetylcholine (ACh) and other substrates is generally small. Q_{10} values from 1.3 to 1.5 are usual. With many preparations, activity is directly proportional to temperature over the range below 35°C, but some experiments have given results consonant with the Arrhenius formulation. Other difficulties concerned in the calculation and interpretation of the standard thermodynamic constants are outlined; and it is concluded that with most preparations the temperature response is modified by complicating factors of an unknown nature.

Heat inactivation of ChE's is rapid at temperatures above 50°C. Fly head preparations are somewhat more sensitive than the ChE's of mammalian blood. Apparent energies of activation for the denaturation are of the order of 40–60 Cal/mole; however, the reaction fails to follow first-order kinetics over longer exposures, and analysis of the data shows a rapid initial phase and a subsequent slower phase. Both processes appear to be of first order. The reason for the biphasic course of denaturation is not known, although it obviously indicates that more than one reaction is involved in the denaturation process.

The ChE's of mammalian serum are in addition reversibly inhibited at temperatures above 40°C. In contrast, all of the reduction in activity observed with fly head ChE is attributable to irreversible denaturation. None of the current hypotheses accounts satisfactorily for all of the anomalies in the temperature response.

The temperature dependence of the reaction with competitive inhibitors, whether reversible or 'irreversible', is in general similar to the response of the enzyme-substrate system, in so far as the scanty data available permit one to judge. Similar complexities are indicated. Some noncompetitive inhibitors at low concentrations combine reversibly with the enzyme, in a process that has a small temperature coefficient. These same inhibitors, at high concentrations, denature the enzyme irreversibly. The energy requirement for such denaturations is of the order of 40–60 Cal/mole, as for denaturation by heat.

In all three areas considered, the need for further study is evident. Little has been done as yet from the point of view of comparative physiology; and there have been no investigations of the temperature dependence of these reactions in the living organism. These gaps will have to be filled, and the anomalies that have come to light must be explained before useful generalizations about the system will be possible.

REFERENCES

1. Abderhalden, E. and H. Paffroth. Beitrag zur Frage der Inkret- (Hormon-) Wirkung des Cholins auf die motorischen Funktionen des Verdauungskanals. *Fermentforsch.* 8: 299–307, 1926.
2. Abdon, N.-O. and B. Uvnäs. A step-photometric method for determining the activity of acetylcholine esterase in blood plasma. *Skandinav. Arch. f. Physiol.* 76: 1–14, 1937.
3. Aldridge, W. N. The inhibition of erythrocyte cholinesterase by tri-esters of phosphoric acid. 3. The nature of the inhibitory process. *Biochem. J.* 54: 442–448, 1953.
4. Alles, G. A. and R. C. Hawes. Cholinesterases in the blood of man. *J. Biol. Chem.* 133: 375–390, 1940.
5. Bacq, Z.-M. Récherches sur la physiologie et la pharmacologie du système nerveux autonome. XIX. La choline-estérase chez les invertébrés. L'insensibilité des crustacées à l'acétylcholine. *Arch. internat. physiol.* 42: 47–60, 1935a.
6. Bacq, Z.-M. La choline-estérase chez les invertébrés. *Compt. rend. soc. biol. Paris.* 120: 247–248, 1935b.
7. Bullock, T. H., H. Grundfest, D. Nachmansohn and M. S. Rothenberg. Effect of di-isopropyl fluorophosphate (DFP) on action potential and cholinesterase of nerve. II. *J. Neurophysiol.* 10: 53–78, 1947.
8. Butterworth, J. Ph.D. Thesis, cited from Davies, 1955. Bristol, England: University of Bristol, 1953.
9. Carlson, L. D. Enzymes in ontogenesis (Orthoptera). XVII. Esterases in the grasshopper egg. *Biol. Bull.* 81: 375–387, 1941.
10. Chadwick, L. E. and J. B. Lovell. The effect of temperature on the activity of fly head cholinesterase. Proc. Tenth Internat. Congr. Entomol., Montreal. In press, 1956.
11. Chadwick, L. E., J. B. Lovell and V. E. Egner. The effect of various suspension media on the activity of cholinesterase from flies. *Biol. Bull.* 104: 323–333, 1953.
12. Clark, A. J., J. Raventós, E. Stedman and Ellen Stedman. Kinetics of cholinesterase. *Quart. J. Exper. Physiol.* 28: 77–86, 1938.
13. Coleman, M. H. and D. D. Eley. Bristol Res. Rep. No. 211, cited from Davies, 1955. March, 1954.
14. Courville, D. A. and W. Ledington. A new method of assay for serum esterase. *J. Biol. Chem.* 190: 575–584, 1951.
15. Davies, J. H. *The kinetics of butyrocholinesterase.* (Ph.D. Thesis.) Bristol, England: University of Bristol, 1955, pp. 1–169.
16. Engelhart, E. and O. Loewi. Fermentative Azetylcholinspaltung im Blut und ihre Hemmung durch Physostigmin. *Arch. exper. Path. u. Pharmakol.* 150: 1–13, 1930.
17. Galehr, O. and F. Plattner. Über das Schicksal des Acetylcholins im Blute. I. Mitteilung. *Pflügers Arch. ges. Physiol.* 218: 488–505, 1928a.

18. Galehr, O. and F. Plattner. Über das Schicksal des Acetylcholins im Blute. II. Mitteilung. Seine Zerstörung im Blute verschiedener Säugetiere. *Pflügers Arch. ges. Physiol.* 218: 506–513, 1928b.
19. Gautrelet, J. and N. Halpern. Antagonisme du curare, du cyanure d'éthyle et de l'hémolymphe vis-à-vis de l'action inhibitrice de l'acétylcholine sur le coeur d'Helix pomatia. Influence de l'ésérine. *Compt. rend. soc. biol. Paris.* 118: 412–414, 1935.
20. Glasstone, S., K. J. Laidler and H. Eyring. *The Theory of Rate Processes.* New York: McGraw-Hill, 1941.
21. Glick, D. Effect of temperature on activity of choline-esterase. *Proc. Soc. Exper. Biol. & Med.* 40: 140–143, 1939.
22. Goldstein, A. The mechanisms of enzyme-inhibitor-substrate reactions, illustrated by the cholinesterase-physostigmine-acetylcholine system. *J. Gen. Physiol.* 27: 529–580, 1944.
23. Goldstein, A. and M. E. Doherty. Properties and behavior of purified human plasma cholinesterase. I. Stability and thermal denaturation. *Arch. Biochem.* 33: 22–34, 1951a.
24. Goldstein, A. and M. E. Doherty. Properties and behavior of purified human plasma cholinesterase. II. Inactivation by mercuric chloride. *Arch. Biochem.* 33: 35–49, 1951b.
25. Johnson, F. H., H. Eyring and M. J. Polissar. *The Kinetic Basis of Molecular Biology.* New York: Wiley, 1954.
26. Kahane, E. and J. Levy. Sur l'hydrolyse diastatique de l'acétylcholine par le sérum. *Compt. rend. acad. sci. Paris.* 202: 781–783, 1936.
27. Kavanau, J. L. Enzyme kinetics and the rate of biological processes. *J. Gen. Physiol.* 34: 193–209, 1950.
28. Kodera, Y. Über das Schicksal des Acetylcholins im Blute. III. Mitteilung. Der Einfluss der Oberflächengrosse auf das Ausmass der Spaltung. *Pflügers Arch. ges. Physiol.* 219: 181–189, 1928.
29. Laurent, P. Influence of temperature on the cholinesterase activity (of the intestine) of *Ameiurus nebulosus,* cited from *Chem. Abstr.* 47: 7681b. *Compt. rend. soc. biol. Paris.* 146: 1523–1527, 1952.
30. Loewi, O. and E. Navratil. Über humorale Übertragbarkeit der Herznervenwirkung. X. Mitteilung. Über das Schicksal des Vagusstoffs. *Pflügers Arch. ges. Physiol.* 214: 678–688, 1926.
31. Means, O. W., Jr. Cholinesterase activity of tissues of adult *Melanoplus differentialis* (Orthoptera, Acrididae). *J. Cell. Comp. Physiol.* 20: 319–324, 1942.
32. Nachmansohn, D., M. A. Rothenberg and E. A. Feld. The *in vitro* reversibility of cholinesterase inhibition by diisopropyl fluorophosphate (DFP). *Arch. Biochem.* 14: 197–211, 1947.
33. Nachmansohn, D., M. A. Rothenberg and E. A. Feld. Studies on cholinesterase. V. Kinetics of the enzyme inhibition. *J. Biol. Chem.* 174: 247–256, 1948.
34. Ormerod, W. E. Hydrolysis of benzoylcholine derivatives by cholinesterase in serum. *Biochem. J.* 54: 701–704, 1953.
35. Pighini, G. Sul dosaggio comparativo della colinesterasi in vari settori del sistema nervoso. *Biochim. e terap. sperl.* 26: 157–159, 1939.
36. Plattner, F. and H. Hintner. Die Spaltung von Acetylcholin durch Organextrakte und Körperflüssigkeiten. *Pflügers Arch. ges. Physiol.* 225: 19–25, 1930.
37. Richards, A. G. and Sping Lin. Proc. Tenth Internat. Cong. Entomol., Montreal. In press, 1956.

38. Roan, C. C. and S. Maeda. The cholinesterase of the oriental fruit fly and its *in vitro* reactions with various insecticidal compounds. *J. Econ. Entomol.* 46: 775-779, 1953.
39. Robert, B., M. Robert and L. Robert. Cited from Davies, 1955. *Bull. Soc. chim. biol.* 34: 183, 1952.
40. Shukuya, R. On the kinetics of human blood cholinesterase. II. The temperature effect upon cholinesterase activity. *J. Biochem.* 40: 135–140, 1953a.
41. Shukuya, R. On the kinetics of human blood cholinesterase. III. Inhibition of cholinesterase by urethane. *J. Biochem.* 40: 535–545, 1953b.
42. Shukuya, R. On the kinetics of human blood cholinesterase. IV. The inhibition of cholinesterase by sodium salicylate. *J. Biochem.* 41: 469–480, 1954.
43. Smallman, B. and L. S. Wolfe. Unpublished data, 1956.
44. Tahmisian, T. N. Enzymes in ontogenesis: choline-esterase in developing *Melanoplus differentialis* eggs. *J. Exp. Zool.* 92: 199–213, 1943.
45. Taysum, D. H. M.Sc. Thesis, cited from Davies, 1955. Bristol, England: University of Bristol, 1952.
46. Vahlquist, B. On the esterase activity of human blood plasma. *Skand. Arch. f. Physiol.* 72: 133–160, 1935.

MECHANISM OF 'HEAT ACTIVATION' OF ENZYMES[1]

Morton N. Swartz, Nathan O. Kaplan and Mary E. Frech,
Massachusetts General Hospital, Boston, Massachusetts, and McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland

There are at least two distinct situations in which the phenomenon of 'heat activation' of an entirely inactive enzyme has been demonstrated. In the first instance the mechanism would appear to be perhaps the initiation of an auto-catalytic process. Bodine *et al.* (1, 3–5) have shown that protyrosinase (a completely inactive form of the enzyme tyrosinase) obtained from grasshopper eggs and purified by ammonium sulfate fractionation can be converted into active enzyme by heating at 60°–70°C. The effect of temperature, however, is not unique. Treatment with various detergents, urea, acetone or the simple addition of extracts from grasshopper embryos similarly initiates the activation of the proenzyme.

In the second instance the activation appears to be related to the destruction or inactivation by heat of a naturally occurring protein inhibitor of the enzyme (8, 12). A necessary corollary to this, of course, is that the enzyme in question be relatively heat-stable. In the present paper we shall dwell exclusively on this latter type of heat activation and describe a group of enzymes that are activated by boiling.

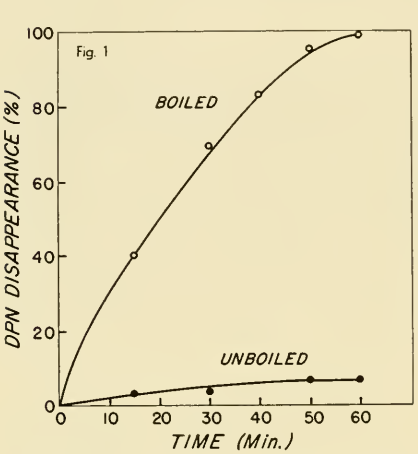
ORIGINAL OBSERVATION

During the course of studies on the pyridine nucleotide content of *Proteus vulgaris* X-19 (ATCC 6380), it was noted that whereas trichloracetic acid extracts of this organism contained considerable amounts of these nucleotides, aqueous extracts prepared from boiled cells contained primarily nicotinamide riboside (NR) and very little diphosphopyridine nucleotide (DPN) or nicotinamide mononucleotide (NMN). This discrepancy was clarified when subsequent experiments demonstrated the presence, in sonic extracts of this organism, of two enzymes (a DPN pyrophosphatase and a 5′ nucleotidase) which were essentially inactive unless the extracts were

[1] Contribution No. 152 of the McCollum-Pratt Institute, Johns Hopkins University. Aided by grants from the National Science Foundation (Grant No. G 2187), the American Cancer Society as recommended by the Committee on Growth of the National Research Council, and the National Cancer Institute of the National Institutes of Health (Grant No. C-2374.C).

placed in a boiling water bath for several minutes. This activation can be seen quite strikingly in figure 1, where the DPN pyrophosphatase activity of the boiled extract is at least 10–15 times that of the unboiled material.

It might be pointed out that these enzymes are the only 'heat-activated' ones found thus far in extracts of *Proteus*. Other enzymes, such as alcohol dehydrogenase and nucleoside phosphorylase are readily inactivated by heating, as one might expect.



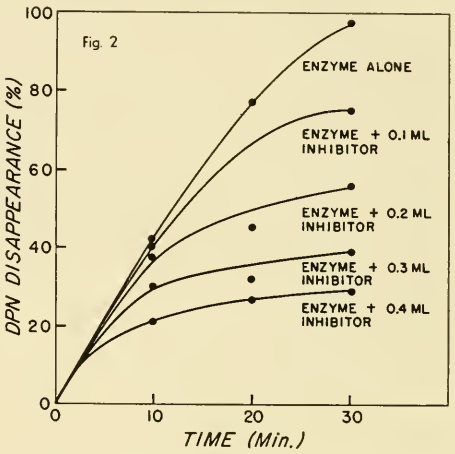


Fig. 1. Activation by boiling of DPN pyrophosphatase. The enzyme preparation was a 1–6 sonicate of *Proteus vulgaris* cells in water. Reaction run at pH 7.5 (tris buffer). DPN remaining at given times was assayed by the alcohol dehydrogenase method. (*Science* 123: 50, 1956.)

Fig. 2. Effect of inhibitor on DPN pyrophosphatase. The enzyme was an 18-fold purified active pyrophosphatase from *Proteus vulgaris*. Inhibitor fraction dialyzed for 18 hours against distilled water. (*Science* 123: 50, 1956.)

DISTRIBUTION OF 'HEAT-ACTIVATED' ENZYMES

Having unwittingly stumbled upon this unusual phenomenon in *Proteus vulgaris*, it seemed of interest to carry out a rough survey to ascertain the extent of this odd reaction in various tissues and microorganisms. The only enzymatic reaction studied was the hydrolytic cleavage of DPN at either the pyrophosphate bond (reaction 1) or at the nicotinamide ribose linkage (reaction 2) where A stands for adenine, R, ribose, P, phosphate, and N, nicotinamide.

reaction 1:

$$\text{A-R-P-P-R-N} \rightarrow \text{A-R-P} + \text{N-R-P}$$

reaction 2:

$$\text{A-R-P-P-R-N} \rightarrow \text{A-R-P-P-R} + \text{N}$$

Mycobacterium butyricum (8), *Bacillus subtilis, Proteus morganii, Proteus rettgeri,* and *Staphylococcus aureus*[2] contain similar enzymes, whereas 30 other microorganisms tested do not exhibit this phenomenon. The 'heat-activated' enzymes from *Mycobacterium* and *Bacillus subtilis* carry out reaction 2 and are true DPNases. Kern (8) has shown that the DPNase of *Mycobacterium* is not inhibited by high concentrations of nicotinamide and does not carry out the exchange reaction between the nicotinamide moiety of DPN and isonicotinic acid hydrazide. The enzymes isolated from the other organisms mentioned above appear to carry out reaction 1 and are organic pyrophosphatases. It is quite remarkable that this number of truly heat-stable enzymes exists, since the screening for the 'activation' was carried out by actually boiling sonic extracts of the organisms. As yet, a search for 'heat-activated' proteinases, sulfatases or β galactosidases has not been rewarding.

Thermophilic bacteria, such as *Bacillus stearothermophilus* studied by Hutner *et al.* (2), would perhaps be a likely source of similar enzymes, but as yet we have no information in this regard. Bacterial spores might be another prospective source (9). Stewart and Halvorson (11) have presented evidence supporting the thermostable-enzyme theory for thermophilism. A racemase which they obtained from spores of *B. terminalis* remained active after exposure to 80°C for 2 hours, whereas the corresponding enzyme prepared from vegetative cells was almost completely inactivated in 15 minutes at the same temperature.

It is interesting to speculate on the possible relationship of the phenomenon of 'heat-activation' to the so-called 'temperature mutants'. In general these mutants of *Neurospora* have little or no activity of a given enzyme when grown at about 35°C whereas at 25°C active enzyme is present (6, 13). However, Yanofsky has found a 'temperature mutant' of *Neurospora* which grows without added tryptophane at temperatures of 30°C or above, but requires this amino acid for growth at lower temperatures (14). Extracts of this particular mutant, td_{24}, are the only ones from a group of 25 mutants at the td locus, which contain tryptophane synthetase activity. Perhaps it is possible that the growth, without added tryptophane, at the higher temperature is related to the destruction, at that temperature, of an inhibitor of tryptophane synthetase.

Thus far none of the higher plant or animal tissues that have been investigated have shown heat-activated DPNases or DPN pyrophosphatases. Most of the information concerning 'heat-activated' enzymes relates to *Mycobacterium* (8) and *Proteus* (12) and the following discussion will concern the enzymes from these sources.

[2] The work on *Staphylococcus aureus* has been carried out by Mr. A. Mildvan in Dr. A. G. Osler's laboratory.

HEAT-LABILE INHIBITORS

Nature of Enzyme-Inhibitor Complex. As has been mentioned, the basis for this apparent 'heat activation' resides in the fortuitous interaction of a heat-labile inhibitor and a heat-stable enzyme. Some extracts of *Proteus vulgaris* contain roughly a 50% excess of free inhibitor, that is, inhibitor not already tightly bound to the enzyme. The effect of this free inhibitor on the active purified DPN pyrophosphatase can be seen in figure 2. Preincubation of enzyme and inhibitor would probably have made the linear relationship between the amount of inhibitor and the degree of inhibition evident earlier in the time course.

The inhibitor is non-dialyzable and has been purified several fold by alkaline ammonium sulfate and ethanol fractionation. The purified enzyme-inhibitor complex contains no detectable ribonucleic acid, and this inactive complex is not activated by ribonuclease. Neither trypsin nor pepsin causes discernible activation of the complex. Acid treatment (pH 1–2) for 10 minutes at 25°C destroys the free inhibitor. Similar treatment inactivates bound inhibitor and in this fashion the enzyme is converted to the fully active form without heating. The enzyme remains active even after the pH is restored to neutrality. The above information suggests that the inhibitor is a protein and that it is probably bound to the enzyme by an ionic linkage rather than by a peptide or similarly strong bond.

Working with *Mycobacterium* which contains roughly a 12-fold excess of inhibitor over enzyme, Kern (7) has been able to purify the protein inhibitor of DPNase quite extensively. Using this preparation he has been able to show that the inhibitor forms an essentially undissociable complex at neutral pH with the enzyme and that the formation of this complex occurs at a measurable rate. As with the *Proteus* enzyme, acid treatment will activate the inhibited enzyme. Unlike the *Proteus* enzyme, however, restoring the pH to neutrality will return the enzyme to the inhibited form. Then, only after boiling is DPNase activity once again found. Thus, like the trypsin-pancreatic inhibitor compound (10) this DPNase-inhibitor complex is reversibly dissociable at acid pH (7).

Heat-lability of Purified Inhibitor. The heat lability of the protein inhibitors of the *Proteus* enzyme and the *Mycobacterium* enzyme is rather different. Kern (7) has shown that while the inhibitor in *Mycobacterium* is markedly heat-labile in crude extracts, the purified inhibitor is surprisingly heat-stable. The heat lability in crude extracts is increased by NaCl but the purified inhibitor remains stable even in the presence of salts. The factor which contributes to the heat lability of this inhibitor in crude extracts of *Mycobacterium* is not as yet known. In contrast, the inhibitor from *Proteus* remains heat-labile even after many steps of purification.

The requirement for high temperatures for complete activation of the *Proteus* enzyme can be seen in figure 3. Inhibited enzyme preparations (enzyme-inhibitor complexes) were heated for two minutes at various temperatures and DPN pyrophosphatase activity was then assayed at 37°. Maximum activity was achieved only after boiling the preparations.

Specificity of Inhibitors. The protein inhibitors obtained from the various bacteria previously mentioned appear to be quite species-specific and there has been no evidence of cross reaction thus far. The inhibitor from *Mycobacterium butyricum* does not inhibit the DPNase from pig brain, *Neurospora crassa*, *Bacillus subtilis* or *Chromobacter violaceum* (7).

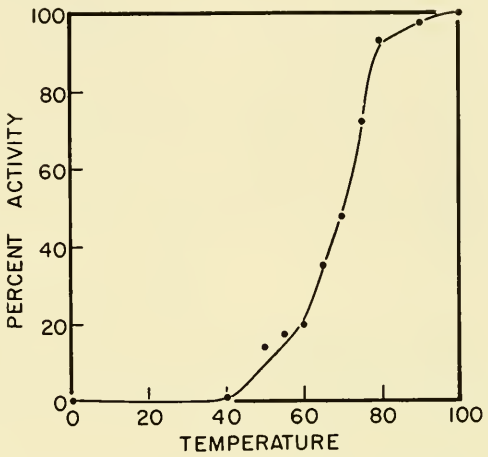


FIG. 3. Heat activation of inhibited DPN pyrophosphatase. The enzyme preparation was a 1–6 sonicate of *Proteus vulgaris* cells in water. DPN splitting was measured by alcohol dehydrogenase method.

Also, the DPN pyrophosphatase of *Proteus vulgaris* is not affected by the inhibitor from *Mycobacterium*.

Further information concerning the nature of the enzyme-inhibitor complex may be gleaned from some studies in which antibodies to the inhibited enzyme of *Proteus vulgaris* were produced in rabbits.[3] These antibodies significantly inhibited purified active DPN pyrophosphatase preparations from the same organism. It is quite likely that the complex dissociated after injection or that the inhibitor was destroyed, exposing the 'active' and antigenic site on the enzyme. Alternatively, it is possible that the antigenic site and 'active' site on the enzyme are contiguous. It is interesting to note that the antibodies to the heat-stable enzyme are themselves heat-labile, i.e. boiling the purified enzyme inhibited by antibody will restore activity. Just as in the case of the inhibitors there ap-

[3] The work with antibodies was done in collaboration with Dr. A. G. Osler, Department of Microbiology, School of Public Health and Hygiene.

pear to be no cross reactions of the antibodies. For example, the antibody to the DPN pyrophosphatase from *Proteus vulgaris* will not inhibit the DPN pyrophosphatase from *Staphylococcus aureus*. Further work is currently in progress in Dr. Osler's laboratory in correlating the properties of the various protein inhibitors and the related antibodies.

Role of Inhibitor-Enzyme Complex. One question that may be raised concerning this inhibitor and the inhibitor-enzyme complex relates to its role in the cell. Does this enzyme occur naturally in the cell in the inactive form or is this an artifact produced by sonic disruption of the cell, allowing the inhibitor and enzyme to interact? In the case of *Mycobacterium butyricum*, no DPNase activity is evident in either whole cells or sonic extracts unless they are boiled. The situation with regard to the DPN pyrophosphatase from *Proteus* is much less clear. Whole cells exhibit pyrophosphatase activity. Sonic extracts are inactive. Heating these extracts produces demonstrable enzymatic activity, approximately 25% greater than that of a comparable whole cell preparation. There is some evidence to suggest that the enzyme in the whole cell is on the cell surface and is different from the enzyme isolated from sonicates. However, at the moment this must remain a moot point.

HEAT-STABLE ENZYMES

Heat-stability of Crude Enzyme Preparations. Although the DPN pyrophosphatase from *Proteus* is surprisingly heat-stable for an enzyme, it is nevertheless inactivated by rigorous and prolonged heating. In figure 4 it can be seen that the activity of the crude extract is maximal after two minutes of boiling, and that after 15 minutes the activity is reduced by 75%. The remarkable stability to heat of this enzyme resembles the stability of trypsin and myokinase.

Reversible Denaturation of Purified Enzymes. The effect of temperature on the activity of the enzymes from *Mycobacterium* and *Proteus* may be seen in table 1*A* and *B*. In each instance the enzymes have been purified about 40–fold. The first step in this purification was boiling, in order to destroy the inhibitor. The enzymatic reactions were then run at the temperatures indicated. The temperature optimum for the DPN pyrophosphatase of *Proteus* is about 50°C, while that of the DPNase of *Mycobacterium* is about 40°C. In both instances the purified enzymes are heat-labile and most of the activity is lost when the reaction is run at 60° or 70°C. However, this inactivation is reversible; for upon cooling the reaction mixtures and incubating them at 37°C, the expected increase in activity is observed.

Factors Affecting Heat Stability of Enzymes. Originally, the 'heat-activated' pyrophosphatase of *Proteus* was demonstrated in extracts that

had been boiled in demineralized water. This finding was quite fortuitous, since subsequently it has been shown that the boiling of crude extracts in buffers such as tris (hydroxymethyl) aminomethane, (tris), has led to inactivation of the enzyme. Further work has revealed several interesting points on the heat stability of this enzyme. The ability of the enzyme to

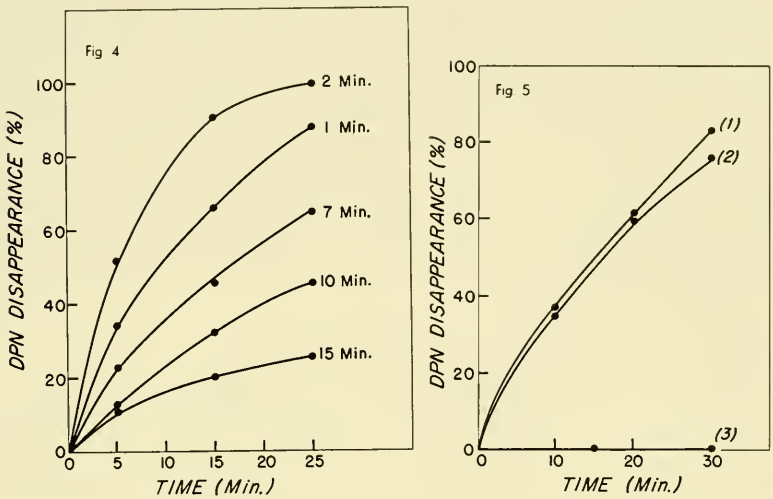


Fig. 4. Effect of prolonged boiling on DPN pyrophosphatase. The enzyme preparation was a 1–6 sonicate of *Proteus vulgaris* cells in water, and assays were carried out in usual fashion.

Fig. 5. Stabilization of enzyme to heat by pyrophosphate. The reaction mixture contained 0.5 ml of crude enzyme preparation that was boiled for 2 minutes, 2 μ moles of DPN, .08 M tris buffer at pH 7.5, and 1 μ mole of $CoCl_2$ in a final volume of 1.5 ml. *Curve 1*, undialyzed enzyme. *Curve 2*, enzyme dialyzed for 18 hours against distilled water, then 2 μ moles of inorganic pyrophosphate added to 0.5 ml of enzyme and boiled for 2 minutes. *Curve 3*, same as *curve 2* except that the pyrophosphate was added to the reaction after the enzyme had been boiled. (*Science* 123: 50, 1956.)

Table 1. Activity of mycobacterium butyricum and of proteus vulgaris at various temperatures

TEMP., °C	UNITS/ML.	TEMP., °C	UNITS/ML.
*A. Mycobacterium butyricum**		*B. Proteus vulgaris*	
20	543	30	200
30	915	40	350
40	1140	50	430
50	882	60	321
60	111	65	104
		70	0

* Data from M. Kern. In press.

withstand boiling requires a dialyzable factor. Extracts dialyzed for 18 hours against distilled water are completely devoid of activity after boiling. As can be seen in figure 5, when inorganic pyrophosphate is added to the dialyzed preparation prior to boiling, essentially full activity is retained. Cobalt is added to the reaction mixtures since this metal or manganese is needed for enzymatic activity and is apparently lost on dialysis. An as yet unidentified organic phosphate compound in the barium-soluble, alcohol-insoluble fraction of trichloracetic acid extracts of fresh *Proteus* cells will also afford protection against high temperatures. DPN, NMN, 5′-adenylic acid (5′-AMP), metaphosphate, 5′-inosinic acid (5′-IMP), TPN, CoA, ATP and ribose-5-phosphate will not replace either this compound or inorganic pyrophosphate. The concentration of inorganic pyrophosphate in *Proteus* extracts is far below the level that affords maximal protection. Thus, it would not appear to be the 'natural' protective factor.

The protection afforded by pyrophosphate applies to the purified enzyme

Table 2. Purified proteus enzyme: protection against boiling by na pyrophosphate

NA PYROPHOSPHATE CONC.	% PROTECTION
1×10^{-1}	81
6.4×10^{-2}	59
6.4×10^{-3}	53
1×10^{-3}	10
1×10^{-4}	0

as well as to crude extracts. In table 2 it can be seen that a high degree of protection is conferred on the purified enzyme by sodium pyrophosphate. It is interesting that the concentration of the latter required is about 18 times the optimum concentration for protection of the crude enzyme. A concentration of pyrophosphate similar to that used with the crude extracts will protect the enzyme when the purified enzyme-inhibitor complex is boiled. The explanation of this is not very clear although it might suggest that the presence of the inhibitor, although unable to protect by itself, might potentiate the effect of pyrophosphate.

A similar protective factor for the *Proteus* enzyme has been found in the supernatant fraction of boiled serum. Liver and yeast extracts do not appear to contain this factor in appreciable amounts.

Previously it had been noted that extracts of *Proteus vulgaris* contained a second 'heat-activated' enzyme, a 5′ nucleotidase. It has been difficult to separate these two enzymes by protein fractionation procedures. However, they appear to be distinct enzymes. The pyrophosphatase has an absolute requirement for either cobalt or manganese whereas the 5′ nucleotidase has no metal requirement. Thus far the inhibitor preparations have

not been purified sufficiently to determine whether the two enzymes have the same or different inhibitor proteins. Inorganic pyrophosphate is needed to protect the 5′ nucleotidase just as it is needed to protect the pyrophosphatase against heat denaturation. In contrast, there appears to be no evident protective co-factor for the 'heat-activated' DPNase of *Mycobacterium*. Also, the heat-labile enzyme alcohol dehydrogenase is not rendered heat-stable by boiling in the presence of pyrophosphate.

The mechanism by which pyrophosphate affords protection is not at all clear. Inorganic pyrophosphate is a substrate for the *Proteus* pyrophosphatase, but it is split at an extremely slow rate. Perhaps, just as the substrate glucose protects hexokinase against inactivation by trypsin, pyrophosphate may analogously protect the *Proteus* pyrophosphatase against heat. However, other substrates for this enzyme afford no protection. Also, this would not explain the protective effect for the 5′ nucleotidase.

SUMMARY

Several 'heat-activated' bacterial enzymes have recently been described. The 'activation' has been dependent on the destruction by heat of an inhibitor protein that is more temperature-labile than the enzyme involved. The most interesting aspect of these relationships may lie in the role of various factors in stabilizing or labilizing proteins to the effects of heat. In the case of the *Proteus* DPN pyrophosphatase, inorganic pyrophosphate or another as yet unidentified compound in extracts of *Proteus* will protect against heat inactivation of the enzyme. In the case of the *Mycobacterium* DPNase, an unidentified factor in crude extracts renders the inhibitor protein sensitive to heat.

The enzymes themselves, once freed of the inhibitors, are not 'heat-activated'. They exhibit the usual Q_{10} that one might expect with enzymes. Thus, the 'activation' is a function of the effect of temperature on a bound inhibitor and not of the effect of temperature in 'exciting' the enzyme molecule itself.

REFERENCES

1. Allen, T. H., O. M. Ray and J. H. Bodine. Enzymes in orthopteran ontogenesis. VI. Autocatalytic nature of *in vivo* formation of protyrosinase. *Proc. Soc. Exper. Biol. & Med.* 39: 549, 1938.
2. Baker, H., S. H. Hutner and H. Sobotka. Nutritional factors in thermophily. *Ann. New York Acad. Sc.* 62: 349, 1955.
3. Bodine, J. H. and L. D. Carlson. Enzymes in ontogenesis (Orthoptera). X. The effects of temperature on the activity of the naturally occurring and other activators of protyrosinase. *J. Cell. & Comp. Physiol.* 16: 71, 1940.
4. Bodine, J. H. and T. H. Allen. Enzymes in ontogenesis (Orthoptera) XV. Some properties of protyrosinases. *J. Cell. & Comp. Physiol.* 18: 151, 1941.

5. Bodine, J. H., T. N. Tahmisian and D. U. Hill. Heat activation, inhibition, and injury of protyrosinase and tyrosinase. *Arch. Biochem.* 4: 403, 1944.
6. Horowitz, N. H. and S. C. Shen. Neurospora tyrosinase. *J. Biol. Chem.* 197: 513, 1952.
7. Kern, M. A protein inhibitor of diphosphopyridine nucleotidase from *Mycobacterium butyricum*. In press.
8. Kern, M. and R. D. Natale. A "heat-activated" diphosphopyridine nucleotidase from *Mycobacterium butyricum*. In press.
9. Militzer, W. et al. Thermal enzymes. *Arch. Biochem.* 24: 75, 1949.
10. Northrup, J. H., M. Kunitz and R. M. Herriott. *Crystalline Enzymes*. New York: Columbia, 1948.
11. Stewart, B. T. and H. Halvorson. Studies on the spores of aerobic bacteria. II. The properties of an extracted heat-stable enzyme. *Arch. Biochem.* 49: 168, 1954.
12. Swartz, M. N., N. O. Kaplan and M. E. Frech. Significance of "heat-activated" enzymes. *Science* 123: 50, 1956.
13. Wagner, R. P. and H. K. Mitchell. *Genetics and Metabolism*. New York: Wiley, 1955.
14. Yanofsky, C. Gene Interactions in Enzyme Synthesis. In: *Enzymes: Units of Biological Structure and Function*. Symposium of Henry Ford Hospital. New York: Acad. Press, 1956.

MYOSIN ATP-ASE ACTIVITY IN RELATION TO TEMPERATURE AND PRESSURE

Karl F. Guthe, *Department of Zoology, University of Michigan, Ann Arbor, Michigan*

The molecular events in muscular contraction are still poorly understood, although the protein myosin has long been regarded as the actively contracting substance. For more than twenty years, the important role of adenosine triphosphate (ATP) in muscle energetics has been recognized. In 1939, the discovery that myosin catalyzes the hydrolysis of ATP (6) was received with great enthusiasm. Although Mommaerts (16) has recently presented evidence against the view that ATP splitting is the immediate source of contractile energy, it is nevertheless clear that myosin's ability to contract is closely linked to its ATP-ase activity (25). Whether contractility is linked to ATP-binding, to ATP-splitting or to a steady-state transference by myosin of the terminal phosphate of ATP is still debated. In any case, ATP-splitting is a useful tool for studying the physicochemical properties of myosin.

Myosin ATP-ase is not a very simple system. Its complexity has been shown in many investigations. Only one enzyme is present, and it removes only the terminal phosphate from ATP, but its activity depends on the relative concentrations of magnesium, calcium, sodium, potassium and hydrogen ions, as well as temperature and pressure. Besides these factors, myosin's ATP-ase activity is also influenced by the presence of the other muscle protein actin. No attempt will be made to summarize all the influences, because there are many recent reviews of this subject (1, 15, 19, 21, 23, 25). Instead, data will be presented to show that myosin undergoes reversible denaturation. Preliminary notes (3, 10) will be followed by a more complete report elsewhere (4, 9).

Reversible denaturation is now a quite familiar concept because it occurs in bacterial luminescence, as clearly shown by the analysis of luminescence in terms of the theory of absolute reaction rates (5, 7). According to this theory (11), the rate of a simple reaction depends on the energy needed to form some unstable intermediate complex. The effect of pressure on the rate depends on the volume change between the reactant and the activated complex, while the effect of temperature depends on the corresponding heat change. These changes may depend on the folding or unfolding of the molecule (11, 22). Since pressure tends to fold

the molecule and temperature to unfold it, the two variables should have opposite effects. Their effects on bioluminescence cannot be explained as effects on a simple rate process, but show that the enzyme is also reversibly denatured. As in any other equilibrium, pressure favors the form with less volume, the native form. A decrease in the rate of bioluminescence may be due either to direct inhibition of the rate process or to the removal of active enzyme by reversible denaturation.

Although myosin ATP-ase activity has generally been regarded as responding to temperature and pressure like a simple rate process (12), these two factors do not have opposite effects. Instead, they both increase the activity of the enzyme. This anomalous behavior is caused by the presence of reversible denaturation (3), as will be shown. Such a configurational change or deformation has previously been proposed in several reaction schemes devised to explain other types of evidence on myosin (16, 19, 24).

HYDROGEN ION EQUILIBRIUM

The experiments are simple. For most of them, myosin was extracted from rabbit skeletal muscle with a dilute salt solution at pH 7.4 (Weber-Edsall solution), giving myosin B. This myosin was then washed twice with pH 6 buffer, which converted it to myosin A, as judged by the loss of magnesium activation. For the experiments on the pH dependence of pressure activation, myosin was extracted with a salt solution at pH 6.3 (Guba-Straub solution). A buffered ATP solution was added to the buffered myosin solution, and the ATP-ase activity was measured by the amount of inorganic phosphate recovered after a certain reaction time. The results were calculated as micromoles phosphate split by one milligram of myosin in one minute. Ionic strength was supplied by sodium rather than the customary potassium salts. ATP concentrations were always above 3 mM, more than ten times the concentration usually needed to saturate the enzyme. This simplifies the theoretical interpretation of the result, since combination of substrate with enzyme was not rate-limiting. No inhibition by excess substrate was noted.

The effect of pressure on ATP-ase activity depends on the pH of the solution (fig. 1). It decreases the activity at a pH slightly below 7, and increases it at alkaline pH. This suggests that pressure affects two different processes in the two pH regions. High pH may reasonably be expected to favor unfolding (reversible denaturation) of the molecule, which pressure will oppose, thus increasing the observed rate at high pH. Near neutrality, reversible denaturation should be small, so that most enzymatic sites are active. The inhibiting effect of pressure at this pH is then a direct effect on the rate of breakdown of the enzyme-substrate

complex, since the substrate concentration is high. This breakdown proceeds with a volume increase of 56 cc/mole, from the size of the observed inhibition. The activity under pressure reflects the pH dependence of the rate process (splitting per active site), and it approaches a direct proportionality to hydroxyl ion concentration, in agreement with Mommaert's measurements of atmospheric rates in his best myosin preparations (17).

To find the pH dependence of reversible denaturation, the observed rates

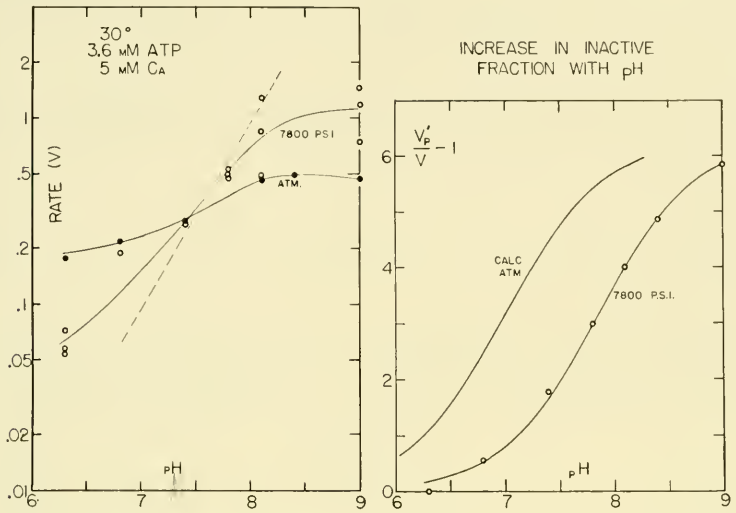


Fig. 1. *Left:* dependence of myosin ATP-ase activity on pH at two pressures (4). The dashed line, which is approached by the data for 7,800 pounds per square inch, corresponds to a direct proportionality between activity and hydroxyl ion concentration. *Right:* corrected relative pressure increment inactivity as a function of pH (4). V_P' is the observed activity corrected for direct pressure inhibition (see text). Points are derived from the smooth curves of the preceding graph. Curves are theoretical titration curves for a monobasic acid. The abscissa is proportional to the fraction of reversibly denatured sites. The left-hand curve is calculated.

under pressure (v_p) are first increased by a constant factor to correct for the direct inhibition of the rate process. The corrected pressure increment ($v_p'/v - 1$) is then plotted against pH (fig. 1, right). For convenience, only the points obtained from the smooth curves on the left are shown. The points follow the curve, which was calculated as a Henderson-Hasselbach titration curve for dissociation of a single hydrogen ion. The good fit justifies the assumptions, and it shows that reversible denaturation proceeds with the loss of a single hydrogen ion with a pK of 7.85 at a pressure of 7800 pounds per square inch. From the size of the pressure activation, the volume increase is 97 cc/mole, and the pK at atmospheric pressure

is about 7. The fraction of denatured sites at any pH is proportional to the upper curve of figure 1, and pressure decreases this fraction to the lower curve, increasing the amount of native enzyme in the system and increasing the observed rate.

Pressure changes myosin activity then for two reasons. First, it increases the relative number of native sites. Second, it decreases the rate of splitting by each active site. Hydroxyl ion has the opposite effect in both cases. At low pH, nearly all sites are active at atmospheric pressure, the decreased rate of splitting predominates, and pressure decreases the observed rate of hydrolysis. At high pH, relatively few sites are native at atmospheric pressure, the pressure increase in the number of native sites more than makes up for the decreased rate of splitting per native site, and pressure increases the observed rate.

According to the usual Michaelis-Menten treatment, the rate, v, is

$$v = \frac{k_2(E)(S)}{K_m + (S)}.$$

At high substrate concentration, $v = V = k_2\,(E)$. If the enzyme is reversibly denatured and only the native form is active, the concentration of native enzyme (number of active sites) must be substituted. If

$$K = \frac{(E_{den})(H^+)}{(HE_{nat})}$$

then

$$v = \frac{k_2(E_{total})}{1 + \frac{K}{(H^+)}} = \frac{\text{const}\cdot T \times (E_{total}) \times e^{-\Delta F^*/RT}}{1 + \frac{e^{-\Delta F/RT}}{(H^+)}}$$

This formula is used throughout.

At constant temperature and enzyme concentration, where only the pressure varies, the ratio of the rate at pressure p to the rate at atmospheric pressure is

$$\frac{V_p}{V} = \frac{\dfrac{e^{-p\Delta V^*/RT}}{1 + \dfrac{K_0 e^{-p\Delta V^*/RT}}{(H^+)}}}{\dfrac{1}{1 + \dfrac{K_0}{(H^+)}}} = \frac{\left(1 + \dfrac{K_0}{(H^+)}\right)e^{-p\Delta V^*/RT}}{1 + \dfrac{K_0}{(H^+)}\,e^{-p\Delta V^*/RT}}$$

if K_0 is the value of K at atmospheric pressure. It is convenient to define

$V'_p = V_p e^{+(p\Delta V^*/RT)}$. This is equivalent to a vertical shift of a logarithmic curve. Then

$$\frac{V_{p'}}{V} - 1 = \frac{\frac{K}{(H^+)}(1 - e^{-p\Delta V/RT})}{1 + \frac{K_0}{N} e^{-p\Delta V/RT}} = \frac{\frac{K_0}{(H^+)} e^{-p\Delta V/RT}(e^{+p\Delta V/RT} - 1)}{1 + \frac{K_0}{(H^+)} e^{-p\Delta V/RT}}$$

Since $K_0 e^{-p\Delta V/RT} = K_p$,

$$\frac{V_{p'}}{V} - 1 = \frac{K_p}{(H^+) + K_p}(e^{+p\Delta V/RT} - 1).$$

This is a Henderson-Hasselbalch curve in shape, with the maximum ordinate equal to $(e^{p\Delta V/RT} - 1)$. The midpoint is pK_p.

CALCIUM EQUILIBRIUM

The preceding experiments were carried out at a constant concentration of calcium ions. The dependence of the pressure effect on pH might

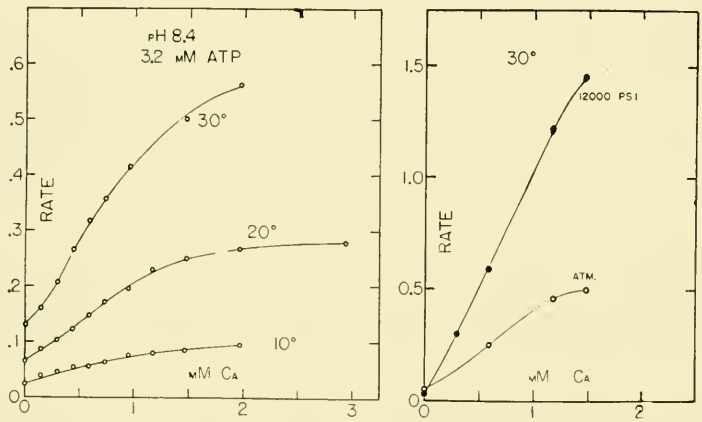


Fig. 2. Dependence of myosin ATP-ase activity on calcium concentration (4). *Left:* activity-concentration curves are the same shape at different temperatures. *Right:* activity-concentration curves are the same shape at different pressures.

simply reflect its dependence on calcium concentration, since calcium activates myosin ATP-ase. The calcium equilibrium cannot, however, be the active-inactive equilibrium we have considered. If the equilibrium constant for the calcium equilibrium were sensitive to temperature and pressure, as that for the hydrogen ion equilibrium is, the ATP-ase activity should reach half its maximum value at a calcium concentration that depends on temperature and pressure. Instead, it is unaltered by temperature (fig. 2) and only slightly changed, if at all, by pressure. The ATP-ase

activity can therefore be studied at constant calcium concentration without fear that the pressure or temperature is changing the amount of calcium-myosin complex through the calcium equilibrium.

TEMPERATURE

The hydrogen ion dependence of the pressure effect is strong evidence for reversible denaturation. The temperature dependence of the ATP-ase activity and of the pressure effect are consistent with reversible denatura-

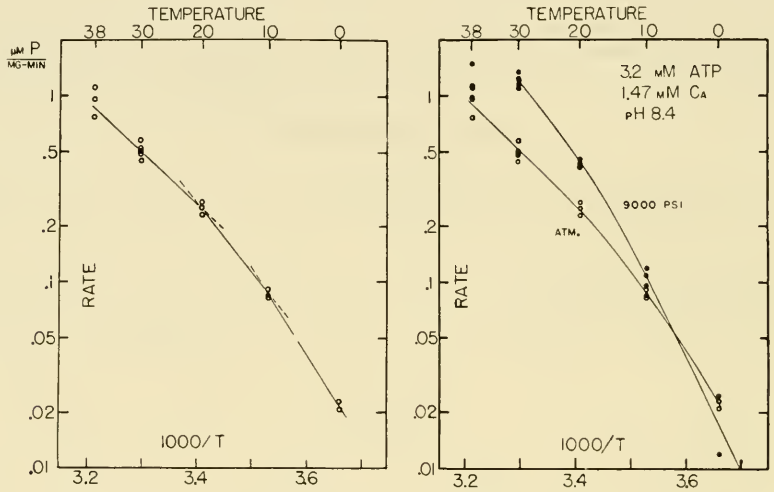


Fig. 3. Dependence of myosin ATP-ase activity on temperature (4). *Left:* activities at atmospheric pressure. Heats calculated for successive straight-line portions of the curve decrease with increasing temperature. *Right:* activities at atmospheric pressure and at 9,000 pounds per square inch.

tion, and reasonable heats can be calculated on this basis. They do not by themselves establish that denaturation occurs.

The activity depends on temperature as shown on the left of figure 3. At first sight, the points fall on a reasonable straight line, as if temperature affects a simple rate process, according to the Arrhenius relation.[1] Closer examination, however, shows that the points depart systematically from a straight line, and the apparent heat of activation decreases as the temperature increases. In bioluminescence and certain other processes (5, 7, 11) a similar but more pronounced departure is caused by reversible denaturation. For myosin ATP-ase, the departure from linearity is small,

[1] They were so reported in a preliminary note(10). The best line corresponds to a heat of activation of 12,400 cal/mole.

but the rate always increases with temperature slightly less rapidly than required by the Arrhenius relation, as expected when denaturation is a significantly rate-limiting factor.

The effects of pressures at different temperatures are also consistent with reversible denaturation. Pressure increases the activity at high temperatures and decreases it or leaves it unchanged at lower temperatures (fig. 3, right). This again suggests that two different processes are affected. If reversible denaturation increases as the temperature increases, most of the enzymatic sites are native at low temperatures. Inhibition at low temperature is then again a direct effect on the rate process, while pressure renatures enough denatured sites at higher temperatures so that the over-all activity is increased.

With the previous values for pK and for the volume changes in the rate process and in reversible denaturation, the temperature data indicate that the heat of reversible denaturation is 12,600 calories per mole and the heat of activation is 31,000 cal/mole for the breakdown of the enzyme-substrate complex. These values were calculated on the assumption that neither volume change is temperature-sensitive. Preliminary experiments show that pressure inhibition at pH 6.3 decreases as the temperature decreases, which indicates that the volume of activation for the rate process, at least, decreases with temperature, like the corresponding volume change in bioluminescence (7). When the temperature-dependence of the volume changes are more accurately determined, the heats will require correction.

PRESSURE

It remains to be shown that the pressure curve is also consistent with reversible denaturation. If activity is plotted against pressure, different experiments show different amounts of increase. A pressure of 12,000 pounds per square inch increases the activity 2½ to 3-fold. The reason for this variation is not clear. Some of it may result from small changes in pH or in other constituents of the solution, such as potassium ion concentration. At present, we are especially interested in the shape of the activity: pressure curve. The different experiments coincide if the increase at each pressure is plotted as percentage of the increase at a pressure of 12,000 pounds/in^2. In figure 4, the value of v_p/v at this pressure was arbitrarily set at 3.0, the observed value in most experiments, and other experiments were corrected on the percentage basis just described.

The data in figure 4 are plotted on a logarithmic scale. If pressure affected only the rate of reaction, the points would be linear, and the volume of activation could be calculated from the slope. At the lower pressures, the slope corresponds to a volume of activation of −40 cc/mole. Above

8,000 lb/in.^{2}, the points begin to fall below the line. The shape of the curve again points to an interplay between two effects of pressure (3). At low pressures, renaturation of active sites dominates, while at high pressures most sites have been renatured and the direct inhibition of enzyme-substrate breakdown becomes more important. The curve in figure 4 was calculated for volume changes of 108 and 63 cc/mole, which fit the data slightly better than the 97 and 56 cc/mole found from the pH data. The fit could undoubtedly be improved by further juggling of numbers, but it is already within experimental error.

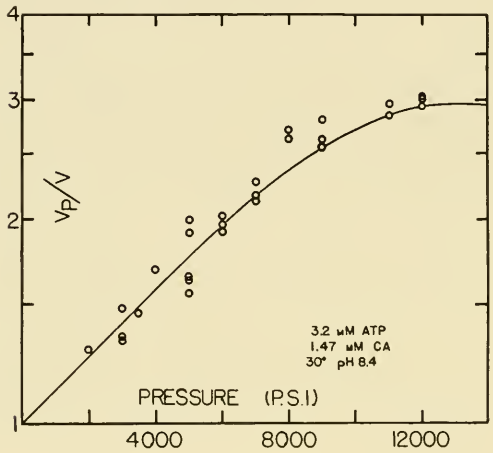


FIG. 4. Pressure increment in myosin ATP-ase activity as a function of pressure (4). For discussion of data points see text. The curve is the calculated increment for a two-fold pressure effect, on enzyme-substrate breakdown and on reversible denaturation.

DISCUSSION

Now that the existence of reversible denaturation is established, the hydrolysis of ATP by myosin involves at least four known single-step reactions. First, the enzyme combines with substrate, which cannot be limiting under the present experimental conditions. The enzyme combines with calcium, which increases the rate but does so independently of temperature and pressure. The reversible denaturation of the enzyme and the breakdown of the enzyme-substrate complex are both sensitive to both temperature and pressure. Several reaction schemes have been suggested (2, 16, 18, 19, 24), into one of which these four reactions may eventually be fitted. The present reversible denaturation may correspond to a suggested deformation or configurational change.

Table 1 lists some of the temperature and pressure characteristics of myosin reactions. The volume change for solation of actomyosin gels (14) is nearly that for reversible denaturation. Pressure favors the sol

form, which may therefore correspond to the present native form. This suggests that myosin in its gel-forming state is not an active ATP-ase. Although the denatured form has been considered completely inactive, for simplicity, the data do not exclude a possible small activity.

The heats and volumes observed by Laidler, Morales and their associates (12, 20) for myosin B ATP-ase agree with our measurements, although our interpretation leads to apparently different values. They have also determined the relative contributions of electrostatic and configurational

Table 1. Heats and volumes determined for the myosin adenosinetriphosphatase system

	ΔV or ΔV^* *cc/mole*	ΔH or ΔH^* *cal/mole*
High substrate (4, 9)		
$E + Ca \rightleftharpoons ECa$	small	small
$ES \underset{\text{Pressure, } H^+}{\overset{\text{Temperature, } OH^-}{\rightleftharpoons}} E + P$	+60	+31,000
$\text{Native} \underset{\text{Pressure, } H^+}{\overset{\text{Temperature, } OH^-}{\rightleftharpoons}} \text{Denatured}$	+100	+12,600
$\text{Myosin B gel} \underset{\text{Temperature}}{\overset{\text{Pressure}}{\rightleftharpoons}} \text{sol}$ (14)	+120	
High substrate (12, 20)		
Phosphate split	−32 to −25[1]	+12,400
Low substrate (12, 20)		
Formation of ES complex	+8 to +23[1]	+8,000

[1] Volume changes become more positive as KCl increases.

effects to the entropy changes in the ATP-ase system (13). Both effects play a significant role at both high and low substrate concentrations, but it is not yet clear how the relative contributions of these two effects are partitioned among the various reactions. Reversible denaturation may be important at both high and low substrate concentrations, since the relation of this reaction to the Michaelis-Menten reaction scheme is uncertain.

The sensitivity of reversible denaturation to pressure, temperature and hydrogen ions has been the main subject of this paper. A sensitivity to other ions might explain the dependence of the apparent volume of activation on KCl concentration (12). The equilibrium may also play a part in other aspects of the myosin problem, such as the pH-dependence of the apparent Michaelis-Menten constant (8) and the existence of deformed

forms of myosin according to light scattering studies (2, 18, 19, 24). The equilibrium may also play a physiological role, since the native and denatured forms are almost equally abundant at pH 7. Its associated heat of reaction may enter the heat budget of the muscle, and its ion sensitivity may fit it for a role in the contractile process.

SUMMARY

Evidence is presented that reversible denaturation plays an important role in the myosin ATP-ase system, as shown especially by the differences in the effects of pressure at different pH. A native site of the enzyme may split ATP, with a heat of activation of about 31,000 cal/mole and a volume increase on activation of about 60 cc/mole. On the other hand, the site may lose a hydrogen ion with a pK near 7 and become reversibly denatured, with a heat of reaction of about 12,600 cal/mole and an increase in volume of about 100 cc/mole. The pK and the heat suggest that reversible denaturation is accompanied by the dissociation of a hydrogen ion from a histidine residue of the enzyme. The large volume increase, however, shows that this dissociation is accompanied by an unfolding of the enzyme or a loss of bound water.

If the pK is 7 and the heat of reaction is 31,000 cal/mole, the entropy of reaction is +10.7 cal/degree-mole. The entropy change for dissociation of an imidazole proton with a pK of 6.8 at 25°C and a heat of dissociation of 7200 cal/mole is −7.1 cal/degree-mole. The corrected entropy change for the protein is then +17.8 cal/degree-mole. This is a small change for a protein molecule, and it indicates that the denatured molecule is not much more disordered than the native.

The analysis shows that the effect of pressure opposes those of temperature and hydroxyl ion in both reversible denaturation and the splitting of the enzyme-substrate complex. That the three factors all increase the measured ATP-ase activity at pH above 7.4 results from the interplay of effects on the two processes. The late discovery of reversible denaturation in the well-studied myosin ATP-ase system appears to be explained by the small heat of denaturation, by the relatively high pressures needed to show departure from a linear pressure relation, by the lack of earlier studies of the pH dependence of the pressure effects, and perhaps by a difference in behavior of the system in sodium as opposed by potassium salt solutions.

REFERENCES

1. Bailey, K. Structural proteins II. Muscle. In: *The Proteins* (ed. by H. Neurath and K. Bailey) II, B: 951–1055. New York: Acad. Press, 1954.
2. Blum, J. J. The enzymatic interaction between myosin and nucleotides. *Arch. Biochem. and Biophys.* 55: 486–511, 1955.

3. Brown, D. E. S. and H. C. Lawler. The activation of myosin-ATP-ase by pressure in the presence of calcium. *Federation Proc.* 5: 13, 1946.
4. Brown, D. E. S., H. C. Lawler, M. P. Carpenter, and K. F. Guthe. The pressure, temperature, and ion relations of myosin ATP-ase. *J. Cell. & Comp. Physiol.* In press.
5. Brown, D. E., F. H. Johnson and D. A. Marsland. The pressure-temperature relations of bacterial luminescence. *J. Cell. & Comp. Physiol.* 20: 151–168, 1942.
6. Engelhardt, V. A. and M. N. Ljubimova. Myosin and adenosinetriphosphatase, *Nature, London,* 144: 668–669, 1939.
7. Eyring, H. and J. L. Magee. Application of the theory of absolute reaction rates to bacterial luminescence. *J. Cell. & Comp. Physiol.* 20: 169–177, 1942.
8. Green, I. and W. F. H. M. Mommaerts. Adenosinetriphosphatase systems of muscle. IV. Kinetics of myosin adenosinetriphosphatase. *J. Biol. Chem.* 210: 695–702, 1954.
9. Guthe, K. F. and D. E. S. Brown. Reversible denaturation in the myosin adenosinetriphosphatase system. *J. Cell. & Comp. Physiol.* In press.
10. Guthe, K. F., H. C. Lawler, M. P. Carpenter and D. E. S. Brown. Activity of myosin and actomyosin ATP-ases in relation to pressure, temperature and pH. *Federation Proc.* 13: 63, 1954.
11. Johnson, F. H., H. Eyring and M. J. Polissar. *The Kinetic Basis of Molecular Biology.* New York: Wiley, 1954.
12. Laidler, K. J. and A. J. Beardell. Molecular kinetics of muscle adenosinetriphosphatase III. Influence of hydrostatic pressure. *Arch. Biochem. and Biophys.* 55: 138–151, 1955.
13. Laidler, K. J. and M. Ethier. Molecular kinetics of muscle adenosinetriphosphatase II. Solvent and structural effects. *Arch. Biochem. and Biophys.* 44: 338–345, 1953.
14. Marsland, D. A. and D. E. S. Brown. The effects of pressure on sol-gel equilibria, with special reference to myosin and other protoplasmic gels. *J. Cell. & Comp. Physiol.* 20: 295–305, 1942.
15. Mommaerts, W. F. H. M. The biochemistry of muscle. *Ann. Rev. Biochem.* 23: 381–404, 1954
16. Mommaerts, W. F. H. M. Investigation of the presumed breakdown of adenosinetriphosphate and phosphocreatine during a single muscle twitch. *Am. J. Physiol.* 182: 585–593, 1955.
17. Mommaerts, W. F. H. M. and I. Green. Adenosinetriphosphatase systems of muscle III. A survey of the adenosinetriphosphatase activity of myosin. *J. Biol. Chem.* 208: 833–843, 1954.
18. Mommaerts, W. F. H. M. and J. Hanson. The effect upon actomyosin of stoichiometric amounts of adenosinetriphosphate regenerated in a coupled enzyme system. *J. Gen. Physiol.* 39: 831–839, 1956.
19. Morales, M. F., J. Botts, J. J. Blum and T. L. Hill. Elementary processes in muscle action: An examination of current concepts. *Physiol. Rev.* 35: 475–505, 1955.
20. Ouellet, L., K. J. Laidler and M. F. Morales. Molecular kinetics of muscle adenosinetriphosphatase. *Arch. Biochem. and Biophys.* 39: 37–50, 1952.
21. Perry, S. V. Relation between chemical and contractile function and structure of the skeletal muscle cell. *Physiol. Rev.* 36: 1–76, 1956.
22. Stearn, A. E. Kinetics of biological reactions with special reference to enzymatic processes. *Advances Enzymol.* 9: 25–74, 1949.

23. Szent-Györgyi, A. G. Structural and functional aspects of myosin. *Advances Enzymol.* 16: 313–360, 1955.
24. Watanabe, S., Y. Tonomura and H. Shiokawa. Mechanism of muscular contraction II. Kinetical studies on muscle ATP-ase. *J. Biochem.* (Japan) 40: 387–402, 1953.
25. Weber, H. H. and H. Portzehl. The transference of the muscle energy in the contraction cycle. *Progress in Biophys. and Biophys. Chem.* 4: 60–111, 1954.

TEMPERATURE-PRESSURE RELATION IN MUSCULAR CONTRACTION

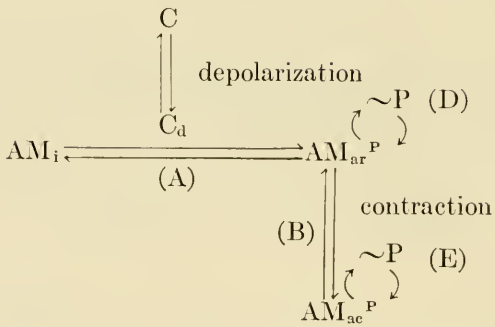
Dugald E. S. Brown, *Department of Zoology, University of Michigan, Ann Arbor, Michigan*

The physico-chemical analysis of muscular contraction in relation to temperature and pressure has been limited by the difficulty of defining shortening and tension in terms of identifiable rate processes and equilibrium changes. In regard to temperature, investigators have been in the perplexing position of being unable to interpret the effects of this factor on any event in contraction in terms of either a heat of reaction, ΔH, or a heat of activation, $\Delta H^{\ddagger}$, or even a net result of both. The same situation exists with respect to the effects of pressure and the volume change of reaction, ΔV, or of activation, $\Delta V^{\ddagger}$.

Recently in studies on myosin ATPase (1) and on the glycerated psoas fiber of the rabbit (2) results have been obtained which offer a clue to a rational interpretation of the action of temperature and pressure in contraction. These results in consequence open the way to a useful comparison of the values of the thermodynamic and rate constants as a step toward the identification of reactions common to myosin ATPase and contractile events in the glycerated fiber and intact muscle. The extensive investigations of Johnson, Eyring and colleagues (3) on a variety of biochemical and cellular processes have clearly shown the usefulness of such a method in the analysis of cellular events. Although a similar approach toward such an analysis of contraction must proceed at present on somewhat limited evidence, its presentation at this time may serve to stimulate critical studies in a direction which appears to deserve further and more intensive investigation.

In considerations of contraction, the concept has developed that the sustained tension in a tetanus or contracture may be pictured as a steady-state condition involving the maintenance of the actomyosin in an active contracted configuration at the expense of concomitant enzymatic and other processes. Schematically the system may be described in formula *(1)* p. 84.

From the studies of Hill (4) on the sartorius of the frog and toad, in which a quick stretch is interposed at various times after stimulation, it is evident that the formation of the relaxed activated unit $AM_{ar}{}^{P}$ begins in the latent period, and the fully 'active state' is reached in the initial one-



(1)

tenth of the contraction phase. In time this coincides approximately with the latency relaxation of Sandow (5) and the development of the alpha state as determined by the abrupt application of high hydrostatic pressures (6). Since $AM_{ar}{}^P$ is fully formed practically by the end of the latent period, shortening and the performance of work depend on reaction B in which relaxed $AM_{ar}{}^P$ passes to the contracted state $AM_{ac}{}^P$.

Hill considered that this reaction was independent of changes in tension or length and proceeded merely as a function of time. In subsequent studies on this reaction in the tetanus by investigating the relation between the rate of shortening under different loads, Hill described the fundamental relation

$$(P + a)v = b(P_0 - P)$$

where P_0 is the maximum tension, P the load, v the rate of shortening, b a constant approximating a rate of energy liberation and a has the dimension of a force. The constants a and b, although critically described, remain to be identified with specific physico-chemical events in contraction.

Polissar (7), in an attempt to give further meaning to the fundamental equation, has proposed a dynamic contractile mechanism in which contractile units in the long ('L') state go over through a reaction sequence (I) to the short ('S') state from which they can return, though by a different pathway (II), to the long state. The reactions (I) predominate in shortening whereas the reactions (II) predominate in relaxing, and a tetanus reflects a steady state of (I) and (II).

The foregoing considerations of Hill and Polissar relating to the sartorius of the frog clearly relate to the development of tension by the fully activated unit $AM_{ar}{}^P$. In other muscles, however, notably the retractor penis of the turtle, there is evidence that the rate of development of tension is governed in part by the rate of development of the active state. Thus Goodall (8) has shown that in this muscle the rate of development of tension in a tetanus is at least ten times slower than the rate of redevelop-

ment of tension following a quick release from maximum tetanus tension where the 'active state' is fully developed.

In the light of these observations, Goodall (9) has reinvestigated the relation between activation and the rate of development of tension. As a result of these studies he has concluded that activation should be considered a function of tension or length as well as time, the dependence being expressed by a rate equation. "The time course of isometric tension development is then determined by a function which measures the initial activation." The discovery that a rate equation describes the dependence of tension on activation is most significant since it presents the possibility of treating contraction in terms of the theory of absolute reaction rates. As Goodall has indicated, it also presents the opportunity of identifying the physico-chemical meaning of the constants a and b in Hill's fundamental equation. In view of Goodall's proposals, the contractile unit in the schema is shown as a phosphorylated activated unit $AM_{ar}{}^{P}$ in which phosphorylation depends on reaction D which, for example, may involve the hydrolysis of adenosine triphosphate by myosin ATPase.

The effects of temperature and pressure on the sequential events in contraction may be conveniently considered in terms of the above schema. In producing contraction, it is supposed that the depolarization of the cell creates a condition, C_d, which causes inactive actomyosin $AM_i{}^{P}$ to pass rapidly to an active contractile form, $AM_{ar}{}^{P}$, which then shortens to $AM_{ac}{}^{P}$. In either the relaxed or contracted states phosphorylases and transphorylases are believed to be active in contributing to the phosphorylation of AM_a.[1]

If the state of depolarization is sustained by repetitive stimulation, the 'active state," in the Hill sense, persists and the maximum tension develops. However, if the depolarization is transient as in the twitch, deactivational processes intervene and the active state rapidly disappears. As a result, the tension developed in a twitch is less than the tension developed in a maximum tetanus.

In terms of the above schema: the steady state of tension requires the active moiety $AM_{ar}{}^{P}$ and depends both on the state of an equilibrium reaction (B) and on the rate of reactions involving the hydrolysis of ATP by myosin ATPase (D). In becoming active through equilibrium (A), it is thought that actomyosin undergoes a molecular reconfiguration to a form capable of shortening and that concomitantly myosin ATPase changes from an inactive to an active enzyme.

As a result of the studies reported by Guthe (10), it seems certain that

[1] The term phosphorylation refers to the phosphate potential as defined by *equation 3*.

a reversible denaturation inactivation of the myosin ATPase establishes the concentration of active native enzyme. In simplified form with reference to myosin alone the overall reaction may be written:

$$\begin{array}{ccc} M_a{}^{ATP} & \xrightarrow{\qquad (R) \qquad} & M_a{}^{ADP} + P \\ \uparrow\downarrow\ (RD) & & \\ M_i{}^{ATP} & & \end{array}$$

in which the steady state production of P depends on reaction (R) catalyzed by the enzyme M_a, while the amount of the catalytically active form of this enzyme is subject to a reversible decrease through the denaturation equilibrium (RD). In the formation of products, the equilibrium (RD) is characterized by $\Delta H = 12{,}000$ cal. and $\Delta V = +120$ cc/mole, while the rate process (R) is characterized by $\Delta H^{\ddagger} = 30{,}000$ cal. and $\Delta V^{\ddagger} = +60$ cc/mole. The effect of increased pressure is to increase the amount of active enzyme, thereby favoring a faster rate of formation of P, while at the same time to retard the rate of the enzyme reaction itself, thereby slowing the formation of P; the net result of pressure is governed by the differences in the values of ΔV and $\Delta V^{\ddagger}$, respectively.

In relation to contraction, the myosin ATPase reaction exhibits two properties essential in the contractile system. First, the reversible denaturation (RD), with its large volume change, offers a device for the conversion of myosin from a denatured inactive form to a native active form with potential shortening properties and simultaneously establishes the concentration of active myosin ATPase. Secondarily, the myosin ATPase, thus controlled, provides for the regulated involvement of high energy phosphate from the pool of phosphate donors and acceptors.

Although the existence of the RD reaction in relation to myosin ATPase is demonstrated (10), it remains to be shown that the RD affects other properties of the myosin molecule such as its reaction with actin. The ready solation of β myosin gels by pressure accompanied by a volume decrease, ΔV, of 120 cc/mole suggests that the RD may be involved in the formation of actomyosin gels (11). Further evidence of the participation of the RD in the contraction of glycerated or normal fibers can be found in the methods of thermodynamic and kinetic analysis.

THE GLYCERATED FIBER

The discovery of the contractility of glycerated fibers by Szent Gyorgyi (12) opened the way to investigations of myosin and actin within the structural organization of a fiber approximating that of living muscle. Physico-chemical studies of contractility in relation to temperature, pH,

and salt concentration by numerous investigators (13) showed that in many ways the responses of these fibers resembled those of intact muscle. The chief obstacle to quantitative studies, however, was the irreversibility of the contraction. This obstacle has been largely removed, first by the discovery of a relaxing factor (14) and later by the discovery that relaxation could be brought about by the addition of creatine phosphate (CP) (15), phospho-enol pyruvate (PEP) (16) and carnosine phosphate (17).

In the course of studies of contractions that were reversible by these agents, fiber preparations were made which exhibited various degrees of contractile activity. Of especial interest was the evidence that prolonged extraction of the glycerated fibers caused the disappearance of the relaxing action of CP, which action could be restored by the addition of ATP-creatine transphorylase to the fiber (18).

A most important property of the glycerated fibers is that, under suitable conditions, auto-oscillations occur (19, 20). In the presence of ATP, Mg, and either CP or PEP, these fibers break into an oscillatory contraction when allowed to develop tension against a torsion lever loaded with a sufficient mass but they fail to do so when the mass is removed. The fact that the lever may be driven at a rate such that the oscillations exceed the free period of the lever indicates that the alternate stretching and releasing of the fiber by the loaded lever affects the capacity of the fiber to develop tension. Such oscillations are a normal occurrence in the flight muscle of certain insects, a fact which gives some confidence that the glycerated fiber may be more than a model of the contractile system and, in fact, has properties very similar to those in living muscle.

In studies on contractions, a highly critical factor is the preparation of the fibers. Lorand's method involves preparation in 50% glycerol and storage for four weeks, then extraction in 0.10 M KCL, 0.01 M Na_2HPO_4 — NaH_2PO_4 at pH 7.0, 4 mM $MgCl_2$, 4 mM ATP and 10 mM phospho-enol pyruvate (21). The resulting fiber develops a sustained contraction on immersion in the testing solution, relaxes on the addition of PEP and is contracted again on the addition of Ca^{++}. In our laboratories, Marcus Goodall prepared what he terms 'A' fibers in a different extraction medium. These were prepared by placing the muscle in a solution of 50% glycerol, 5 mM histidine buffer at pH 6.8, and 2 mM NaCN for a period of two to four weeks. The primary characteristic of Goodall's fiber is that on immersion in the reaction mixture of pH 5.6 at 20°C the fibers contract, but then fully relax. Subsequently these will be referred to as n3 fibers.

Tension and pH. In a series of studies with the n3 fibers, tension development as a function of pH, ATP, CP, temperature and pressure was investigated (22, 2). The central phenomenon turns out to be the pH

dependence of tension. When the relaxed fiber is immersed in 0.15 M succinate buffer, 5 mM $MgCl_2$, and 10 mM ATP, tension develops as a function of pH along a sigmoid curve beginning about pH 5.6 and reaching a plateau at pH 6.8 (fig. 1*A*). The relation is described by

$$Y = \log_{10}[y/(1-y)] = (\Delta H + p\Delta V)/2.3RT + 3(pH - pK) \quad (2)$$

where y is the fractional tension, pK is the pH for half tension, and the factor (n) of 3 indicates that three active sites are involved per unit tension.

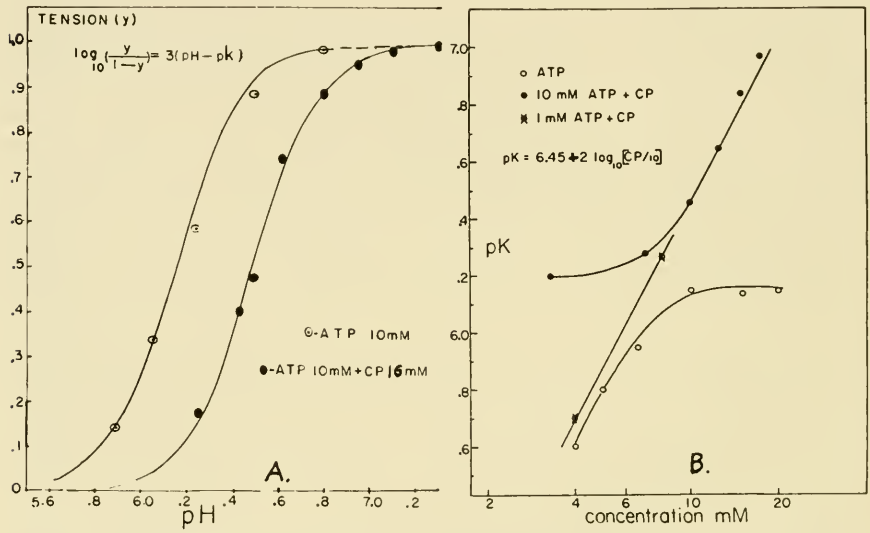


FIG. 1. *A:* Tension in the glycerated rabbit psoas fiber in relation to pH. *B:* Variation in pK in relation to the concentration of adenosine triphosphate and creatine phosphate.

The addition of 16 mM creatine phosphate, a relaxing factor, to the system shifts the curve to the right along the pH axis, increases the pK to 6.5, but leaves the value of n unchanged. Conversely, the addition of 5 mM AMP decreases the pK with a resulting shift to the left. In all instances, however, n retains the value of 3.

In respect to creatine phosphate, the pK is defined as

$$pK = 6.45 + 2 \log_{10} (CP)/10 \quad (3)$$

where 6.45 is the pK for 10 mM CP (fig. 1*B*). It may be noted that here $n = 2$, indicating a two-fold increase in pK per mole of phosphate. If ATP alone is used n also equals 2.

The unique property of this type of fiber is the pronounced sensitivity

to pH as evidenced by $n = 3$. In fibers which develop and sustain the maximum tension with only ATP added, and which are insensitive to CP, n is close to unity (the n1 fibers). Goodall and Andrew Szent Gyorgyi (15) observed in earlier studies that, with CP as a relaxing factor, an extreme sensitivity to pH existed. This suggests $n = 3$ may only obtain when a relaxing factor is present.

In these experiments pH may be considered in the role of an activator inducing the formation of an activated unit with three active sites. Recently Moos and Lorand (21) have studied the induction of contraction by calcium in fibers relaxed by phospho-enol pyruvate at pH 7. On analysis, their data show that here also three active sites are involved per unit tension.

The important fact in these studies is the constancy of n with either pH or Ca^{++} as an activator. With pH as an activator, n remains constant even though the pK value undergoes extensive changes when the concentration of ATP, CP or AMP is varied. The chief effect of these agents, therefore, is to shift the tension curve along the pH axis in a direction and to a degree determined by their effects on the pK. In regard to tension, the significant concept develops that at constant pH the tension is altered primarily by changes in the pK. Thus in the presence of a suitable concentration of ATP, an increase or decrease in the concentration of CP may cause either an increase or a decrease in tension. Since these phosphorylated compounds are involved in enzymatic reactions, their action on tension is subject to modification by those chemical and physical agents which influence enzymatic kinetics. The shift in pK of the activated unit as a whole thus becomes the focal point through which a variety of phosphate donors and acceptors influence tension. Conceptually this is equivalent to considering tension as dependent on a phosphate potential as defined by equation *3*.

Temperature. The variation in tension with temperature has been investigated on standard n3 fibers both in the pH range above 6.8 where the tension is maximal and independent of pH and in the lower range where fractional tensions exist that depend on pH (fig. 1*A*) (22). At all values of pH from 6.15 to 7.3, equal tensions develop depending on the temperature, a higher temperature being required the lower the pH (fig. 2*A*). In the pH range above 6.8 the tension decreases curvilinearly with decrease in temperature but at lower pH values, for example pH 6.15, the curvilinear relation persists only to about 20°C. and thereafter tension decreases linearly with temperature (fig. 2*A*-1).

In the n1 fibers the above relations are modified. Although behaving similarly to the above at pH values higher than pH 6.8, the tension at pH 6.15 varies linearly with temperature from 0° to its maximum at 30°C. In both types of fiber, the linear and curvilinear components are present,

the main difference being that in the n3 fiber the curvilinear component persists within the lower pH range. The tension in this fiber below pH 6.8 thus depends on two separate mechanisms whose effects are additive and which are influenced to a different extent by temperature. In effect, the component that appears to be linear provides a base line upon which the curvilinear component is superimposed. When this base line is taken into account, the curvilinear component bears the same relation to temperature throughout the entire pH range. Plotting $\log_{10}$ [y/(1 − y)] against the reciprocal of the absolute temperature yields a straight line whose slope

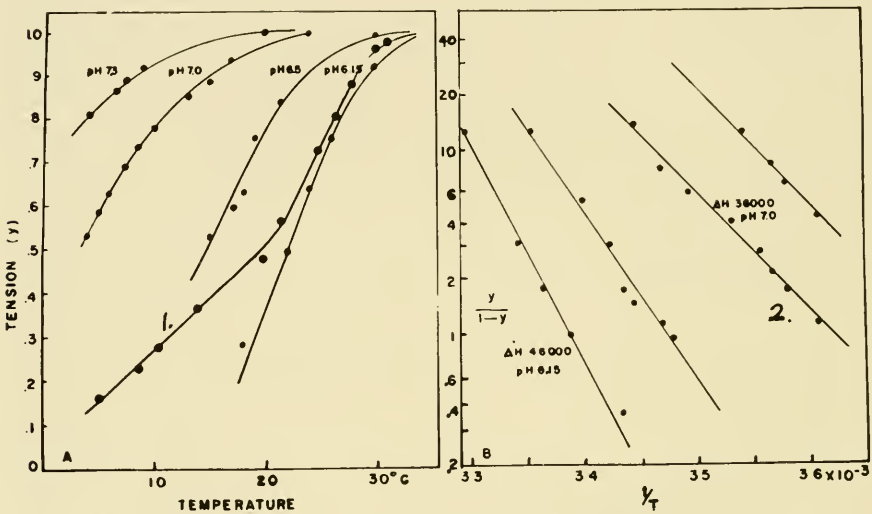


Fig. 2. *A*: Tension in relation to temperature and pH in the glycerated psoas fiber. *B*: Arrhenius plot of $\log_{10}$ [y/(1 − y)] for data of fig. 2*A*.

indicates a ΔH of 36,000 cal. (fig. 2*B*-2). Since as shown in equation 2 the activated unit involves three active sites, the ΔH for the pK per active site should be 12,000 cal.

The dependence of the pK on temperature may be obtained directly by plotting the pH at which half-tension occurs against the temperature. The relation is linear, yielding a $\Delta H^{\ddagger}$ of about 12,000 cal., which confirms the above interpretation of the large ΔH of 36,000 cal.

As mentioned in the preceding section, tension in n1 fibers varies linearly with temperature at pH 6.15 but in the usual curvilinear manner at pH 7.0. It seems clear that quite different processes control the tension-temperature relation at the two pH values. In addition, the fact of their separation by small pH changes indicates a difference in their pK values. Possibly the straight line relation represents an action of temperature on

the capacity of the contracted unit $AM_{ac}{}^{P}$ to sustain tension without returning to $AM_{ar}{}^{P}$. As yet, a linear tension-temperature dependence of this sort has not been observed in normal muscle but this may be due to the failure to use a sufficiently low intracellular pH.

Pressure. In the discussions which follow in this section, the data on pressure all pertain to one temperature, 20°C.

Compression of the relaxed n3 fiber at pH 5.6 with only ATP present causes the development of tension proportional to the pressure, the contraction being sustained until the pressure is removed. The tension in-

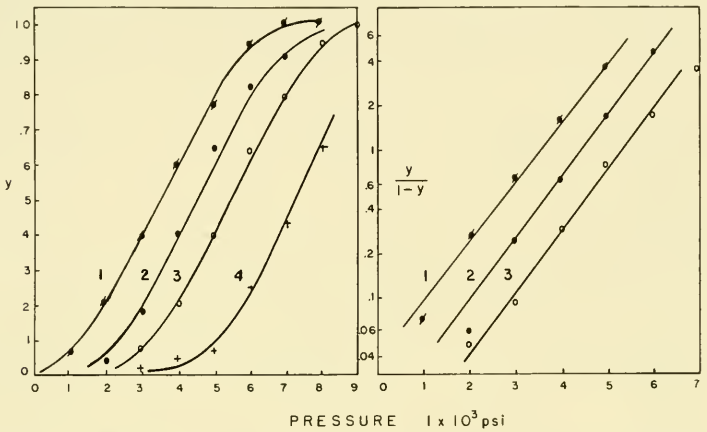


FIG. 3. *A:* Tension induced by pressure in the glycerated psoas fiber and in retractor penis muscle of the turtle. *B:* Log_{10} [y/(1 − y)] in relation to pressure. *Curve 1,* 10mM ATP, pH 5.6; *curve 2,* contracture tension in R. penis of turtle; *curve 3,* 10mM ATP plus 16mM CP, pH 6.4; *curve 4,* 10mM ATP plus .16mM CP, pH 5.6, temperature 20°C.

creases with pressure along an S-shaped curve, reaching an upper limit at a pressure above 8,000 psi (fig. 3*A*). When $\log_{10}$ [y/(1 − y)] is plotted against pressure a linear curve results whose slope is $\Delta V/2.3$ RT and ΔV is −350 cc/mole (fig. 3*B*). Allowing for three active sites per unit tension in accordance with *equation 1,* this would give a ΔV of −120 cc/mole per unit change in pK. In line with the interpretation of the temperature dependence, it is concluded that pressure is acting on equilibrium (A), the formation of $AM_{ar}{}^{P}$ proceeding with a decrease in volume.

In regard to the reaction (A), the sensitivity to pressure depends on ATP and CP (fig. 3*A*). When only ATP is present in the reaction mixture, a pressure of 3,600 psi is required for the development of half maximal tension. With the addition of CP at the same pH of 5.6, the system becomes less sensitive to pressure and a pressure of 7,200 psi is required for half

tension. If the pH is raised to 6.4 with ATP and CP present, the system becomes more sensitive to pressure. In view of these large changes in sensitivity, it is possible that a situation could exist relevant to substrate concentration and pK wherein pressure would have no effect over the range of physiological interest, i.e. up to 10,000 psi.

The foregoing remarks apply to fully relaxed n3 fibers. In the partially contracted fibers at pH 6.8, tension increases with pressure, reaching a maximum at 3,500 psi but thereafter decreases. In contrast, at pH 7.0 where the fiber is fully contracted, pressure always causes a fall in tension with a ΔV of 180 cc/mole (fig. 4A-3). This is interpreted as being due to an inhibition of the phosphorylation equilibrium (D) with a reduction in volume of 60 cc/mole per active site or 30 cc/mole with respect to the

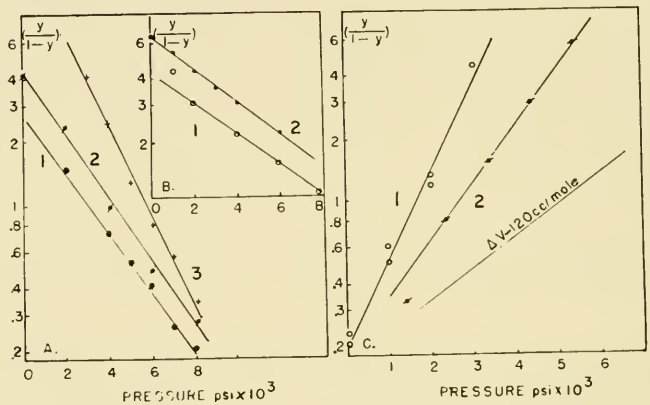


FIG. 4. Log_{10} [y/(1 − y)] in relation to pressure. For description see text.

phosphate ion. This may represent the dissociation of the phosphorylated complex.

In the response at pH 6.8, where pressure first causes contraction and then relaxation, the actomyosin is only partially activated. As a result, the tension at any pressure depends both on the activation equilibrium (A) involving a volume decrease of 350 cc/mole in the formation of the activated unit and on the phosphorylation equilibrium (D) involving a volume increase of 180 cc/mole in maintaining the contracted state.

In n1 fibers, which develop a sustained tension at pH 7.2, the tension-pressure relationship also has a ΔV of 180 cc/mole which remains constant from pH 6.15 to 7.2. In these fibers there is no sign of the activation equilibrium and the fall in tension is most likely due solely to an inhibition of the phosphorylating mechanism (D). As mentioned earlier, the tension-temperature relation for these fibers at pH 7.0 is controlled by reactions characterized by a ΔH of 36,000 cal., while at pH 6.1 tension decreases

linearly with temperature. In both cases the tension depends on the activated unit with three active sites and there is good reason to conclude that the inhibition of phosphorylation does not cause $AM_{ar}{}^{P}$ to pass to the inactive form. The fact that the phosphorylation reaction can act either simultaneously with or independently of the activation equilibrium shows clearly that the systems are distinct and it would be expected that they have different pK values.

In fibers prepared in a manner different from those discussed thus far, namely, by soaking for four weeks in 50% glycerol before testing, tension decreases with pressure in accordance with a volume change of 120 cc/mole (fig. 4*A*-1). These fibers also develop a sustained tension and, like the n1 fibers, are relatively insensitive to pH, indicating that the system is fully activated. The prolonged soaking has evidently created a condition permitting a new mechanism to assume control.

In β myosin gels (11) compression causes solation with a reduction in volume of 120 cc/mole. Pressure also decreases the strength of the cortical gel of the sea urchin egg with a similar decrease in volume (23). In considerations of the cortical gel Johnson *et al.* (3) pointed out that its reaction to pressure could be accounted for in terms of an equilibrium between a native globular and an unfolded fibrous form of the cortical protein. The tension-pressure relation may be similarly interpreted, the decrease in tension resulting from the conversion of the fibrous protein to the globular form with a reduction in volume of 120 cc/mole. In terms of the schema, this would be equilibrium (B), $AM_{ac}{}^{P}$ going to $AM_{ar}{}^{P}$.

In summarizing the results on the fiber, tension depends on three interrelated, but discrete events represented by reactions A, B and D, any one of which may, under suitable conditions, regulate tension.

The equilibria have different pK values and, judging from the effects of CP and ATP, it would be expected that the pK is highest in equilibrium (A) and smallest in equilibrium (B). Thus a fiber relaxed by CP would contract on removal of the CP, the pK for D with respect to ATP then taking over, to be replaced finally by the pK for B on the depletion of ATP or other phosphate donors.

MUSCULAR CONTRACTION

In regard to muscle, the pressure contracture may be readily compared to the pressure induction of contraction in the fiber. The tetanus tension presents a situation where the muscle is apparently fully activated and tension is dependent solely on the state of the activated units. The twitch presents a new situation for here the discrete contractile events occur sequentially. Following stimulation, depolarization produces the activated state C_a, which persists for a short period; actomyosin undergoes activa-

tion and shortening begins but it is soon terminated by the deactivation process. Although presenting experimental difficulties due to the speed of events, the twitch presents the ideal situation for investigating the activator system in relation to the formation of the active contractile complex necessary for the development of tension.

Pressure Contracture. When striated muscle (retractor penis of turtle) is hydrostatically compressed to above 4,000 psi, tension develops, reaching a maximum which is sustained until the pressure is removed (fig. 5) (24, 25, 2). The development of tension begins immediately on compression, but after an abrupt decompression the contraction continues for a brief period before relaxation ensues. The contraction is not accompanied by a

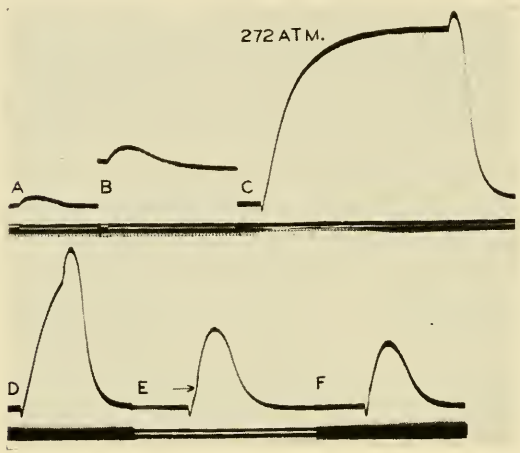


FIG. 5. The pressure contracture of retractor penis of turtle at 10°C, employing abrupt compression and decompression with 4,000 psi. *A:* control twitch. *B:* twitch superimposed on contracture at 2,000 psi. In remaining myograms the pressure is applied abruptly and as abruptly removed progressively earlier in the contraction.

propagated action potential and in its main characteristics satisfies Gasser's critieria (26) for a contracture.

The maximum contracture tension increases with pressure along an S-shaped curve, reaching an upper limit at pressures exceeding 8,000 psi equal to the maximum tetanus tension. The relation is similar to that obtained on the glycerated fiber (fig. 3*A*), and when subjected to the same analysis $\log_{10}[y/(1-y)]$ varies linearly with pressure, with a ΔV of -350 cc/mole (fig. 3*B*, 4*C*-2). As yet, a decrease in tension in the contracture at higher pressures with a resulting optimum in the tension curve has not been observed at pressures up to 12,000 psi.

The contracture also resembles the glycerated fiber in that the minimum pressure necessary to produce the contractures is influenced by chemical agents, in this instance the concentrations of K^+, Ca^{++} and Cl^- in the bath. With 4 mM K^+ and 2 mM Ca^{++} the contracture begins at 500 to 1,000 psi, with 2 mM K^+ and 2 mM Ca^{++} at 3,000 to 3,500 psi, while at

1 mM K^+ and 1 mM Ca^{++} the first sign of contracture is at 7,500 to 8,000 psi.

The withdrawal of Cl^- with substitution of sucrose also increases the sensitivity to pressure. In the retractor penis muscle a contracture develops at normal pressure in proportion to the amount of Cl^- withdrawn. With 25 mM Cl^- remaining, the tension in the Cl^--induced contracture is about 30% of the maximum tension. When pressure is then applied, a superimposed pressure contracture appears at 250 psi with half maximum tension being reached at 3,000 psi. In contrast, a pressure of 9,000 psi is required to produce half tension in the control, although otherwise the tension-pressure curve is the same (27) (28).

Another case of sensitization to pressure is that resulting from stimulation. In the claw muscle of *Callinectes sapidus* compression to 3,000 psi fails to produce a contracture. However, if the muscle is then stimulated under this pressure a twitch develops which relaxes only about 25%, the tension being sustained at the high level until the pressure is removed (29). On the basis of the results with the glycerated fiber, the appearance of the contracture would be attributed to a lowering of the pK. Moreover, since it seems certain that a stimulus initiates some positive changes to compensate for the loss of K^+, it seem appropriate to propose the establishment of a new balance in the phosphate donor and acceptor system.

As indicated above, the pressure contracture in muscle resembles in its major characteristic the pressure induction of contraction in the glycerated fiber. In both, the contraction involves a decrease in volume of 350 cc/mole and in both the pressure sensitivity is considerably modified by chemical agents. It is proposed therefore that the contracture results from the conversion of inactive AM_i to active $AM_{ar}{}^P$ (equilibrium A) with a reduction in volume of 350 cc/mole.

Tetanus. The maximum tetanus tension is generally considered as a 'steady state' condition in which the muscle is maintained in the fully 'active state' by repetitive stimulation. If the period of stimulation is brief, the contraction is rapidly reversible, but continued stimulation leads to various secondary situations in which the tension falls, relaxation is delayed and a fatigue contracture or even rigor develops. In the light of the data on the glycerated fibers, these sequential secondary phenomena would be assigned to changes in pK due to new metabolic conditions arising from the prolonged stimulation. In the present context only the brief, reversible tetani will be considered.

Temperature and pH. The maximum tetanus tension of striated muscles increases with temperature, reaching an upper limit which is then sustained over a considerable temperature range (30). With stigmatic electrodes, the tension decreases at temperatures above 20°C unless the

period of exposure to these higher temperatures is brief. However, with stimulation by the uniform longitudinal field method of Csapo and Goodall (31), which in effect eliminates the propagated disturbances as a factor in contraction, the tension in the retractor penis of the turtle or the sartorius of the frog is sustained without decrement to 30°C (32), and even higher.

For any species, the tension-temperature relation is set on the temperature scale in relation to the physiological temperature range of the species but for any poikilotherm the position is subject to change by a

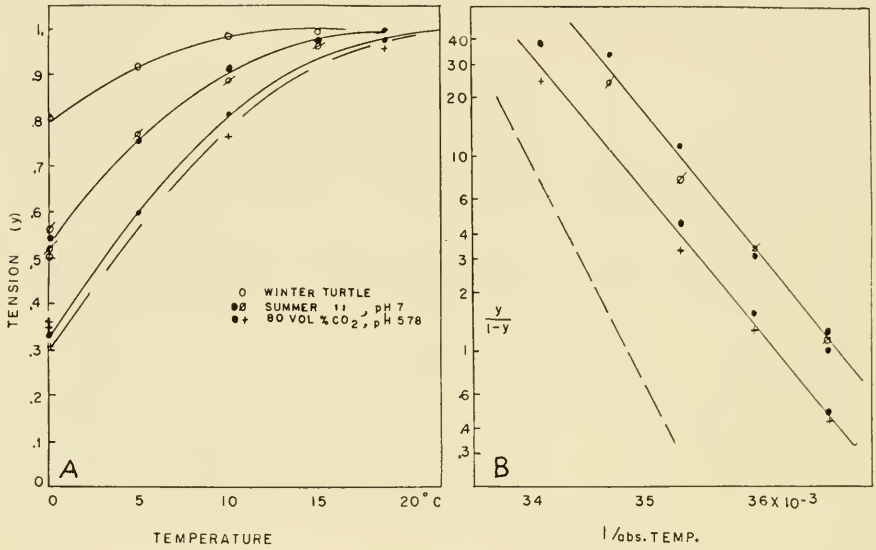


Fig. 6. *A:* Tetanus tension in retractor penis of the turtle in relation to temperature, pH. *B:* Same data as 6*A*. Broken line indicates curve with a ΔH of 46,000 for comparison.

period of temperature acclimatization (33). In our laboratories this phenomenon has been observed in the retractor penis of *Chrysemys picta* (fig. 6*A*). In the muscle of a turtle acclimatized at 5–10°C for four months, about 80% of the plateau tension is developed at 0°C. However, after a similar period of acclimatization at 20°–25°, the curve is shifted to a higher temperature range with the result that at 0°C only 50% of the plateau tension develops.

Factors other than acclimatization can modify the position of the curve on the temperature scale. For example, if the intracellular pH is reduced by equilibrating the muscle with 80% CO_2, the tension-temperature curve is displaced into a higher temperature range with the result that the tension

at 0°C is only 30% of the maximum, which maximum is now attained at about 25°C.

The tension-temperature relation for the tetanus is curvilinear and resembles that obtaining above pH 6.8 in all types of glycerated fibers studied. Letting y equal the fractional tension and plotting $\log_{10}[y/(1-y)]$ against the reciprocal of the absolute temperature, a straight line results with ΔH of 36,000 cal. (fig. 6*B*). This ΔH is the same as that for the fiber under conditions where tension depends on the degree of phosphorylation of the activated unit $AM_{ar}{}^{P}$ with ΔH of 12,000 cal. per active site.

As in the glycerated fiber, a decrease in pH shifts the tension curve into a higher temperature range. On the other hand, the curve is shifted to a lower temperature by acclimatization at low temperatures. In the light of the results on the fiber, this latter phenomenon could be attributed, within a given species, to a decrease in the pK for tension consequent on a new balance in the phosphate donor-acceptor system.

Pressure and Tension. The relation between tetanus tension and pressure is quite complex. Cattell and Edwards (34) reported insignificant changes in maximal tetanus tension on compression. In our laboratories this observation has been confirmed but, in addition, either an increase or a decrease of about 2% per 1,000 psi or no change at all has been observed. In a frog sartorius at 3°C where a contracture appears at 3,500 psi, tetanus tension decreases with pressure in accordance with a volume decrease of 60 cc/mole (fig. 4*B*-1). The b constant of Hill's fundamental equation, calculated from these myograms, is also reduced by pressure with a volume decrease of 60 cc/mole (fig. 4*B*-2).

Under certain conditions where the contracture does not appear until 7,500 psi, the tetanus tension may be unchanged or slightly increased under pressure. Apparently in brief tetani, pressure tends both to induce contraction (equilibrium A) and to inhibit tension (equilibria B and D) indicating that in the maximum tetanus the actomyosin is not entirely in the active form. It is probable that in a prolonged tetanus, the cellular changes would exclude equilibria (A) and (C) and that tension could then come under the control of equilibrium (B) and diminish with pressure with a volume decrease of 120 cc/mole.

Isometric Twitch. For purposes of comparison with the glycerated fiber the relevant aspects of the twitch are the tension-temperature relation in respect to pH and pressure and the influence of pressure on activation. For several reasons the auricular muscle of the turtle is ideally suited for such studies. The slow speed of contraction renders the relatively abrupt application of pressure a simple matter while the long refractory period excludes the possibility of a re-excitation resulting from the abrupt

changes in pressure. In addition, the treppe phenomenon makes possible a study of temperature and pressure on the activation process per se.

In a resting auricle stimulated only for testing (35) the tension increases with temperature and reaches an upper limit which may be maintained up to 30°C provided the exposure to the higher temperature is brief (fig. 7*A*-1). An auricle responding in this manner may be conveniently designated as 'non-treppe.' In contrast, the tension in a resting heart, which may be termed a 'treppe heart,' rises to the upper limit at 9°C, but thereafter decreases progressively with a rise in temperature (fig. 7*A*-2). At 9°C the tensions in the treppe and non-treppe hearts are identical. In the treppe heart repetitive stimulation at a suitable frequency causes an increase in tension at all physiological temperatures above that of the optimum at 9°C. At each of these higher temperatures the maximum tension resulting from repetitive stimulation approximates

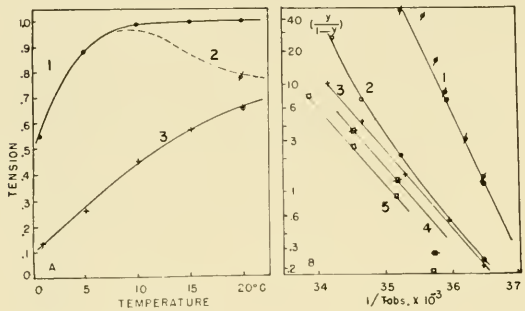


FIG. 7. *A:* Isometric tension in relation to temperature in turtle auricular muscle: (1) non-treppe heart, (2) treppe heart, (3) treppe heart with 80% CO_2. *B:* $\text{Log}_{10}\,[y/(1-y)]$ for auricular tension in relation to temperature. For description see text.

that obtained at the optimum at 9°C, i.e. approximates that of a non-treppe heart (36, 37). As will be indicated subsequently, the treppe phenomenon relates to the activator mechanism, and may be omitted from the immediate considerations of tension.

In the non-treppe heart, the tension-temperature relation resembles that for the tetanus tension in striated muscle. In both, a constant tension is maintained over a wide range of temperatures. In the tetanus this is attributed to the fact that the 'active state' is sustained long enough for the maxmum tension to develop at each temperature. A similar explanation may be put forward for the heart, from which it would follow that in the non-treppe heart the fall-off in tension above the optimum temperature would result from a termination of the 'active state' before tension development has been completed.

In both the treppe and non-treppe heart the tension-temperature relation is similar up to the optimum temperature (fig. 7*A*-1 and 2). Since the duration of the 'active state' is sufficient for maximum tension at the

optimum temperature, it would be supposed that it would also be sufficient at lower temperatures. In this event the tension at these lower temperatures would depend merely on the capacity of the activated units to develop tension. The tension-temperature relation should be the same as that for the maximum tetanus of striated muscle. This, however, is not the situation.

In the Arrhenius plot of $\log_{10}$ [y/(1 − y)] the data for the turtle auricle deviate from a clearly linear relation (fig. 7*B*-1) at the higher temperatures. In the turtle the curve to the maximum covers such a small temperature range that the measurements are subject to considerable error. Recently, however, Dawson and Bartholomew (38) described the tension-temperature relation in the heart of the desert iguana (*Dypsosaurus dorsalis*). In this heart, tension in the twitch rises from 20% of full tension at 2°C to a maximum full tension at 28°–30°C. The Arrhenius plot of these data is similar to that for the turtle and establishes clearly the non-linearity of the relation (fig. 7*B*-2).

A decrease in intracellular pH by exposure to 80% CO_2 as in the case of the tetanus shifts the tension curve of the twitch as in the case of the tetanus toward a higher temperature (fig. 7*A*-3). Moreover, again as in the tetanus, the Arrhenius plot of $\log_{10}$ [y/(1 − y)] for the twitch now yields a straight line whose slope indicates a heat of 36,000 cal. (fig. 7*B*-3).

The same shift in the tension-temperature relation results from compression. Under a constant pressure in the range from 4,000 to 8,000 psi, the Arrhenius plot is linear above 5°C and also indicates a heat or energy of 36,000 cal. (fig. 7*B*-4, 5). Thus, at a low intracellular pH or at high pressure, the contractile system reacts as if all of the actomyosin were in the active form and is being controlled with respect to temperature primarily by equilibrium (D).

Below 5°, however, the tension curve at high pressure deviates from linearity. Referring to the control curve at normal pressure, the tension may be considered as decreasing more rapidly with temperature than would be so if only equilibrium (D) were in control. This would be the result if the duration of the 'active state' at low temperatures was insufficient for the full development of tension. There is the possibility, therefore, that both low pH and high pressure prolong the 'active state' sufficiently so that the tension dependence again comes under essentially the sole control of equilibrium (D). the effect of such a situation in regard to tension would be that at 0° the inhibition by pressure would be less than expected since pressure would be tending to augment tension by prolonging the 'active state' as well as inhibiting the development of tension by the active complex.

Pressure. The characteristic action of pressure on the treppe heart is to reduce the tension at low temperatures and increase it at high temperatures (39, 40). When the relation between tension and temperature is examined in detail (fig. 8) it is found that the general effect of compression is to displace the tension curve to a higher temperature range. At normal pressure, the tension rises with temperature to a maximum at about 9° and thereafter diminishes. At progressively higher pressures,

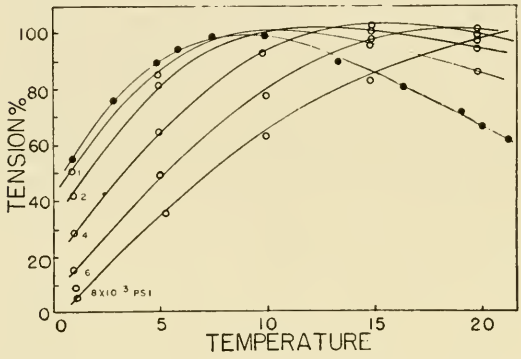


Fig. 8. Auricular tension in relation to temperature and pressure.

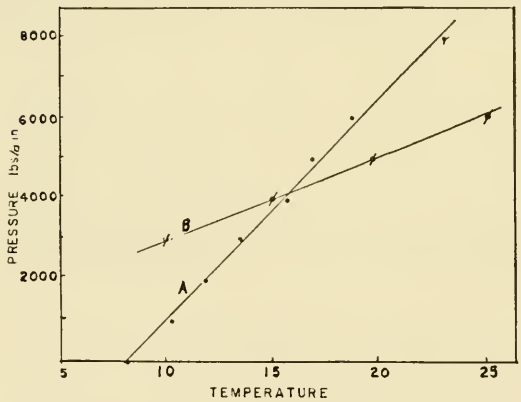


Fig. 9. Temperature and pressure necessary to produce (A) the maximum tension in the auricle, and (B) to produce recession of the cleavage furrow in the sea urchin egg by solation of the cortical gel.

the maximum occurs at higher temperatures, but despite this shift the maximum tension developed remains the same. An upper limit of tension thus exists which is attained at a specific combination of temperature and pressure. A plot of the temperature-pressure relation for equal and maximum tensions is linear (fig. 9*A*), the slope of the curve being proportional to the entropy change of the system which could be calculated provided the ΔV were known.

For purposes of comparison, a similar relation is shown in fig. 9*B* relating to the temperatures and minimum pressures which cause reces-

sion of the cleavage furrow in the sea urchin egg. Here the action is on the cortical gel of the egg which is solated by pressure to a degree depending on the temperature. If, in both muscle and the cortical gel, the decrease in tension results from the conversion of a fibrous form of protein to the globular form with a decrease in volume of about 120 cc/mole, then the entropy change in the muscle would be about three times that for the cortical gel. The difference might turn out to depend on the fact that contraction in muscle involves a protein complex with three active sites while the gel has but one.

In regard to the pressure effect at constant temperature, two situations in the treppe heart are critical. First, at 5°–8°C as shown by the temperature dependence, the 'active state' is apparently sufficiently prolonged to permit maximum tension to develop and here pressure inhibits tension. Second, and in contrast, at 20°C the tension is reduced and compression increases the tension to a level equaling that of the maximum at 9°C and atmospheric pressure.

In regard to the inhibition at 5°C the plot of $\log_{10}[y/(1-y)]$ against pressure is linear and the decrease in tension proceeds with a decrease in volume of 120 cc/mole (fig. 4*B*-2). This is attributed to the conversion of fibrous to globular protein in equilibrium (B).

At 20°C, pressure is remarkably effective in increasing the tension. In experiments designed to study this pressure relation, hearts which gave only 20% of the maximum tension at 20°C were prepared. These hearts permitted measurements over a considerable range of tensions. The tension data, again plotted as $\log_{10}[y/(1-y)]$ against pressure, are linear and show that the change in tension, which under these conditions is in the direction of an increase rather than a decrease, proceeds in accordance with a volume change of 710 cc/mole (fig. 4*C*-1). At first glance this appears to be an unreasonable value, but it deserves consideration in the light of recent results on the production of contractures by withdrawal of chloride (28) and the addition of potassium (41), respectively. In both types of contractures induced by ionic changes, the relation between tension and ion concentration is described by $\log_{10}[y/(1-y)] = n \log [\text{ion}]$ where for Cl^- and K^+, n is between 5 and 6. Here, the value of n is twice as large as the value of n with respect to pH or Ca^{++} in the glycerated fiber. In muscle, however, both chloride withdrawal and potassium excess cause depolarization of the cell membrane. The view is currently held that the contracture is a consequence of the depolarization of the cell. If, in these ion-induced contractures, depolarization produces an activator C_d, each unit of which induces the formation of two $AM_{ar}{}^{P}$ units, a value of $n = 6$ would result.

In the case of the cardiac twitch, it is therefore suggested that the

depolarization is coupled to the formation of the activated unit $AM_{ar}^{I'}$ (equilibrium A) in this manner. Thus, with a ΔV of 120 cc/mole for the pK per active site, the ΔV of 360 cc/mole would govern the formation of each activated unit and a ΔV of 720 cc/mole would result from two such units being activated per unit of activator C_a.

Earlier in the present paper, the decrease in tension at the higher temperature was attributed to the duration of the 'active state' being insufficient to permit the full development of tension. The compression

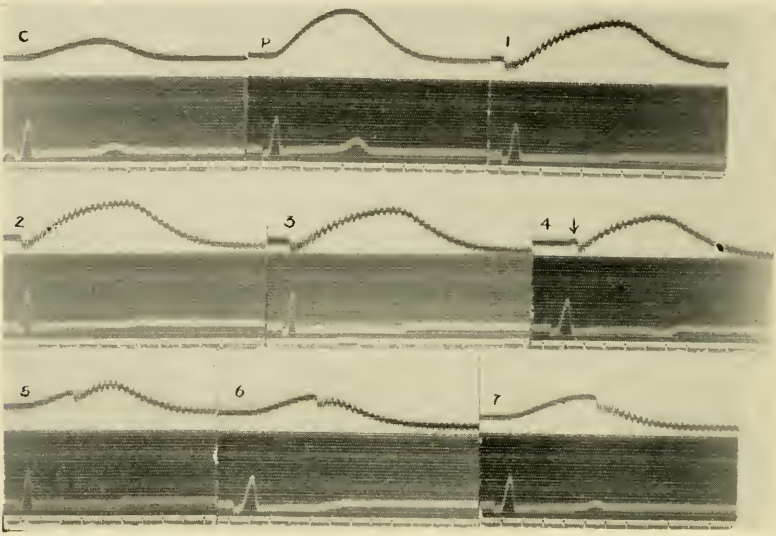


FIG. 10. Myograms of the auricular muscle of *Pseudomys elegans* illustrating the effects of abrupt compression to 4,000 psi at various times during the contraction cycle. Temperature 20°C. Myogram C, recorded at atmospheric pressure; P at 4,000 psi, the presure being applied 15 seconds before stimulation. In remaining records the time of abrupt compression is indicated by the displacement of the tension record.

data, however, indicate that the larger tensions depend on equilibrium (A) and hence the development of the 'active state.' The development of this state is generally considered to occupy only the latent period plus the initial one-tenth of the contraction phase. The question at issue, therefore, is whether a compression during this brief period is sufficient to produce the large contractions appearing under a sustained pressure.

In a muscle at 20°C the tension developed under a sustained pressure is 2.5 times greater than in a control at atmospheric pressure (fig. 10). However, when the muscle is stimulated and then abruptly compressed to this pressure after only one-fifth of the tension has developed, the resulting contraction is only about 12% greater than the

control (fig. 10-4, 11). It is clear that, during a brief period following stimulation, a process occurs which is augmented by pressure and which basically determines the tension to be developed. The pressure-effective period begins at stimulation and is 80% completed by the end of the latent period.

The converse of this experiment involves compression of the muscle prior to stimulation followed by abrupt decompression when about one-fifth tension has developed. The result of such a decompression is that further development of tension ceases, while the tension already developed is sustained until relaxation intervenes at the normal time. This experiment shows that although compression during the latent period is necessary in order to augment the tension, compression also must be maintained

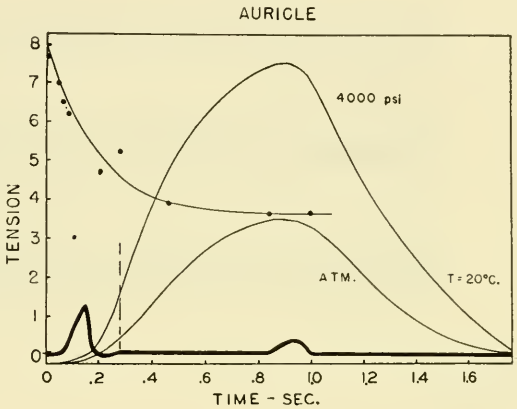


FIG. 11. A plot of the total tension developed when the abrupt compression is applied progressively later in the contraction cycle. Compression at the broken line and thereafter sustained has little effect on tension. Conduction time, 0.1 seconds.

during the contraction phase for full tension to develop. From this result, it is inferred that the conditions which are set up by pressure during the latent period, and which are essential for a greater tension, disappear rapidly on decompression. In view of the large decrease in volume of 710 cc/mole, it is concluded that the process involved is the production and disappearance of the activator C_d. In Goodall's terminology, C_d establishes the activation charge which determines the rate of development of tension and, in the twitch, the duration of the active state.

At low temperatures where the major effect of pressure is one of inhibition, the use of an abrupt compression provides information on the sequence of events. At 5°C the twitch under a sustained pressure is smaller and relaxation is slowed (fig. 12*C*, *P*). An abrupt compression, interposed after the initial one-fifth of the contraction is completed, causes a small abrupt fall in tension, a smaller peak tension finally developing (fig. 12-1). As evidenced by the larger tension developed under a sus-

tained pressure, the compression at this temperature is also increasing the degree of activation (fig. 12-*P*, as compared to fig. 12-1). The additional fact revealed, however, is that the state of tension, once developed, is abruptly reduced by compression with a reduction in volume of about 120 cc/mole (fig. 12-2). The effect is reversible, a decompression at peak tension causing a very rapid redevelopment of tension (fig. 12-4).

Treppe Phenomenon. In the preceding discussions the treppe heart is dealt with. The phenomenon of treppe itself deserves consideration. The low tension in such a heart at 20°C can be increased by repetitive stimulation and at a suitable frequency attains the plateau tension. This

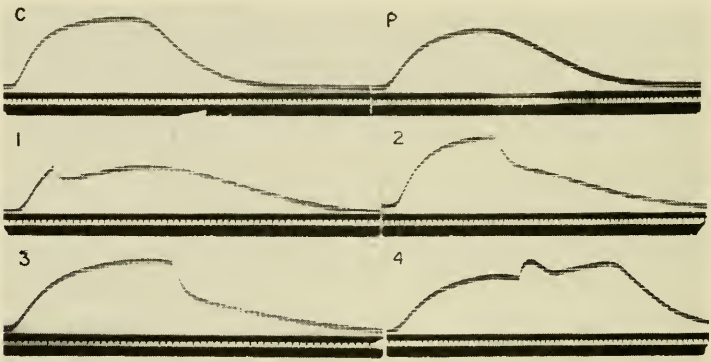


Fig. 12. Myograms of the turtle auricle at 5°C showing the effects of an abrupt compression applied during the contraction cycle. Myogram C, the control at atmospheric pressure; myogram P recorded at 4,000 psi. In myograms 1, 2 and 3 an abrupt compression to 4,000 psi is interposed as indicated. Myogram 4 recorded at 4,000 psi with an abrupt decompression interposed at the time of peak tension. The slow and prolonged contraction results from a re-excitation of the tissue by the decompression.

has long been recognized as the treppe, or staircase, effect. Recently, Twente (37) has shown that such a muscle in full treppe is practically insensitive to pressure. Moreover, the resting muscle could be brought to full treppe tension within 10 rather than the usual 75 contractions, provided it was compressed during only the initial one-fifth of each contraction phase. In the light of the preceding results, it would appear that the treppe phenomenon depends on changes in the amount of activator C_a that has been formed at the onset of contraction. This seems particularly important in relation to the possible locus of action of Ca^{++} and other agents which eliminate treppe. It is noteworthy that in muscles which normally do not produce treppe, and where at 20°C the plateau tension develops, pressures up to 10,000 psi do not alter the tension. In this case activation either does not play a significant part or is diminished

to a level just equalling the pressure inhibition of tension development by the activated unit.

Isometric Twitch of Skeletal Muscle. The information regarding the sequence of events in the isometric twitch of skeletal muscle is so extensive that the preceding results on cardiac muscle which are not so fully known may be considered to better advantage in this context. In the beginning it may be emphasized that the response of cardiac muscle to abrupt changes in pressure confirms earlier results on the retractor penis of the turtle and on the sartorius of the frog (*R. pipiens*) (43). In the retractor penis, appli-

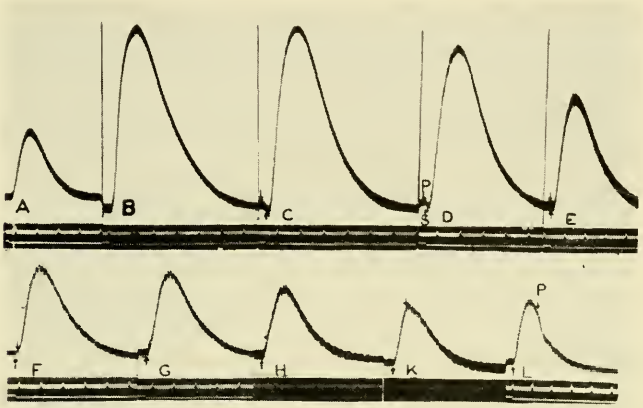


FIG. 13. Myograms illustrating the augmentation in tension produced by an abrupt compression to 272 atmospheres during the contraction cycle. Retractor penis muscle of the turtle. Temperature 20°C. Myogram A recorded at atmospheric pressure. Myogram B recorded at 272 atmospheres, the pressure being applied 15 seconds before stimulation. In remaining records, the moment of compression is indicated by the upper arrow, P, while the moment of stimulation is indicated by the lower arrow, S. Time scale equals 0.04 second per division.

cation of a pressure of 4,000 psi prior to stimulation and maintained throughout the contraction causes a considerably larger twitch which is also somewhat prolonged (fig. 13*A*, *B*; fig. 14). In contrast, an abrupt compression applied when one-tenth of the tension has developed at atmospheric pressure and the pressure maintained thereafter produces a twitch not particularly different from the control (fig. 13*F*, 14). The converse of this experiment, done at 3°C and employing an abrupt decompression, shows that compression for only the first one-tenth of the contraction results in a twitch tension 95% of that developed under a sustained pressure (44). From this it was concluded that in this brief period pressure sets up a condition which determines the duration of the

'active state' of Hill and hence the amount of tension developed. In earlier publications, this event was called the 'alpha process' (46) and it was indicated that its time course was of the order of that for the contraction potential of Bishop and Kendall (45). It is the state, resulting from depolarization, which sets the level of activation and determines the duration of the active state. In view of the results on the glycerated fiber and the heart muscle, the alpha process would be interpreted as depending on the production and disappearance of the activator C_d.

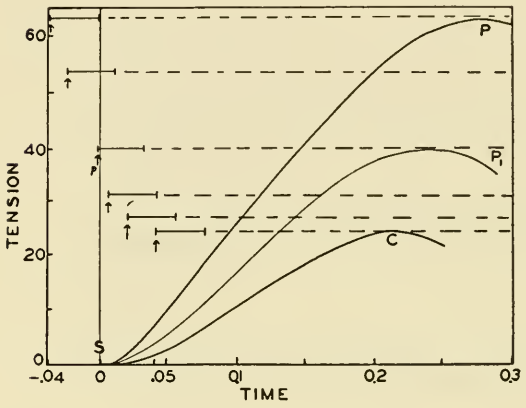


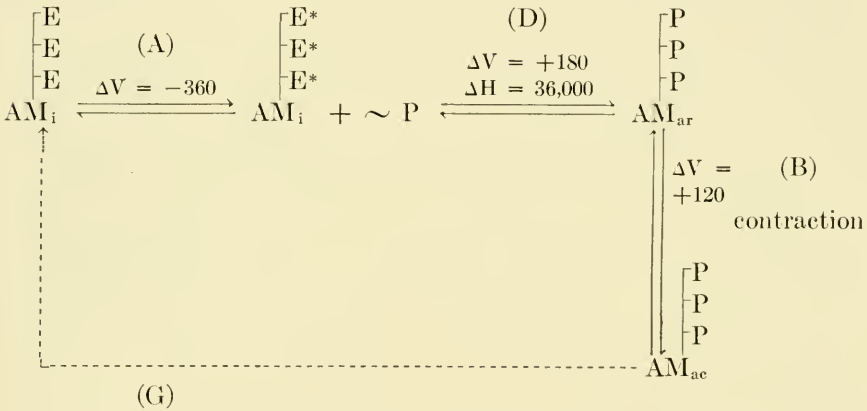
FIG. 14. Curve C: the myogram recorded at atmospheric pressure. Curve P: the myogram recorded at 272 atmospheres, the pressure being applied 15 seconds before stimulation at S. Curve P_1: the myogram recorded following an abrupt compression at the moment of stimulation and sustained thereafter; the total tension attained is indicated by the broken line, the moment of compression by the arrow p, the total pressure being attained within the interval delimited by the solid line in brackets following the arrow. The remaining broken lines indicate the total tension attained following compressions at the moments indicated by the arrows.

DISCUSSION AND SUMMARY

An understanding of the temperature-pressure relation in muscular contraction involves the identification of discrete, measurable, contractile events and an interpretation of these events in physical and chemical terms. The basis for the physico-chemical approach lies, in general, in the temperature-pressure relation of isolated components, such as actomyosin, enzyme systems essential to contraction and extracted muscle fiber systems. In this paper an attempt has been made to present ways in which the influence of temperature and pressure may be useful in revealing important aspects of the contractile system.

In muscular contraction the recognizable events, considered in sequence, are: *1)* the production and disappearance of an activator (C_d) with which the alpha process is identified; *2)* the conversion of inactive actomyosin to the activated contractile form; *3)* the development of tension by the activated unit.

The glycerated fiber systems differ from muscle in that the activator, such as pH or calcium, is under the control of the investigator whereas in muscle the production of the activator is a consequence of the depolarization of the cell. The alpha process, described in the high pressure studies, is considered to depend on the time course of the activator cycle. Experimentally it is measured in terms of tension which, however, merely reflects the activation charge set up at the beginning of the contraction. In the auricular muscle of the turtle it turns out that the formation of the activated contractile complex as the result of a stimulus proceeds with a reduction in volume of 720 cc/mole. In relation to other events in contraction, this large volume decrease occurs coincidentally with the latency relaxation and the development of the 'active state.' In terms of the schema below, the very large volume reduction is considered to arise from a situation in which each mole of activator catalyzes the formation of two activated units, each unit being activated undergoing a reduction in volume of 360 cc/mole or 120 cc/mole per active site.



In the preceding it has been considered that the activator reaction (A) and the phosphorylating reaction (D) are necesary for the formation and contraction of the activated unit. In such a system the activation charge would be proportional to the number of activated units produced by reaction (A) and also to the phosphate potential per unit as set by reaction (D). Referring to the schema, reaction (A) proceeds with a decrease in volume of 360 cc/mole and is augmented by pressure, while reaction (D) proceeds with an increase in volume of 180 cc/mole and is inhibited by pressure. It is clear from the opposite signs of the ΔV values that the reactions are quite different. The difference is further borne out by the fact that the activation requires time to build up and is governed by a rate equation. It may be supposed that reaction (A),

with its large volume decrease, activates three enzymatic sites which then catalyze the phosphorylation reaction which proceeds with a decrease in volume.

In contractions of the glycerated fiber produced by pressure at pH 5.6 the tension developed is controlled by reaction (A) with no indication of an inhibitory reaction which would be expected if reaction (D) were implicated. At pH 7.0, however, the inhibition of tension via reaction (D) becomes a significant factor, particularly at the high pressures. Apparently the two reactions have a sufficiently different pK value to eliminate the action of pressure on reaction (D) at the lower pH. In the pressure contracture in muscle there is no indication of reaction (D), and reaction (A) appears to be in control over the physiological range of pressures. In contrast to the extensive action of pressure in contracture, the maximum tetanus tension may be decreased, increased or unchanged by pressure. In the auricular muscle of the turtle at 5°C tension at the peak of the twitch is inhibited by pressure with a volume decrease of 120 cc/mole. Such a variable effect of pressure on maximum tension raises the possibility that where the pressure effect is small tension may have escaped from the controlled reactions (A) and (D) and be governed in respect to pressure by factors inherent in reaction (B), such as the fibrous state of the protein.

In regard to temperature, the major simplification which appears is that the tension-temperature relation is governed by the pK of the active sites. Since a major factor in determining the pK is the phosphorylation reaction (D), the heat constant represents an energy of activation, $\Delta H^{\ddagger}$ and has a value of 12,000 cal. This is an important consideration since the value of 36,000 cal., resulting from the three active sites being involved per unit tension, would be quite incompatible with results of thermal studies on the muscle if it represented a heat of reaction. In the schema a ΔH of 36,000 is assigned to reaction (D). As regards reaction (A), it appears to be temperature-independent. In reaction (B), however, there is the possibility, as indicated by the linear temperature dependence of the glycerated fiber at pH 6.15 that this reaction has a ΔH of about 6,000 cal.

Another possibility which has not been dealt with in this paper is that a decrease in tension may involve the return of the contracted unit to the inactive form by another pathway (G). In the light of Polissar's and Goodall's consideration this requires examination, particularly in relation to certain irreversible effects of pressure on tension in the glycerated fiber.

REFERENCES

1. Brown, D. E. S., K. F. Guthe, H. C. Lawler and M. P. Carpenter. The pressure, temperature and ion relations of myosin ATPase. In press.

2. Goodall, M. C. and D. E. S. Brown. Reversal of relaxing mechanism in muscle fiber systems by hydrostatic pressure. *Nature*, 178: 1470–1471, 1956.
3. Johnson, F. H., H. Eyring, and M. J. Polissar. *The Kinetic Basis of Molecular Biology*, New York: Wiley, 1954.
4. Hill, A. V. The earliest manifestation of the mechanical response of striated muscle. *Proc. Roy. Soc., London, B*, 138: 339–348, 1951.
5. Sandow, A. Latency relaxation and a theory of muscular mechano-chemical coupling. *Ann. N. Y. Acad. Sci.* 47: 895–929, 1947.
6. Brown, D. E. S. The regulation of energy exchange in contracting muscle. *Biological Symposia*, III: 161–190, 1941.
7. Polissar, M. J. Physico-chemical model of contractile mechanism. *Am. J. Physiol.* 168: 766–781, 1952.
8. Goodall, M. C. Rate of activation in smooth and skeletal muscle. *J. Cell. & Comp. Physiol.* 46: 351, 1955.
9. Goodall, M. C. Activation and non-linear elasticity in muscle. *J. Gen. Physiol.* In press.
10. Guthe, K. F. and D. E. S. Brown. Reversible denaturation in the myosin-adenosine triphospate system. In preparation.
11. Marsland, D. A. and D. E. S. Brown. The effect of pressure on sol-gel equilibria with special reference to myosin and other protoplasmic gels. *J. Cell. & Comp. Physiol.* 20: 295–305, 1942.
12. Szent Györgyi, A. Free-energy relations and contraction of actomyosin. *Biol. Bull.* 96: 140–161, 1949.
13. Hasselbach, W. and A. Weber. Models for the study of the contraction of muscle and of cell protoplasm. *Pharmacol. Rev.* 7: 97–118, 1955.
14. Marsh, B. B. The effect of adenosine triphosphate on the fibre volume of a muscle homogenate. *Biochim. et biophys. acta*, 9 (3): 247–260, 1952.
15. Goodall, M. C. and A. G. Szent Gyorgyi. Relaxing factors in muscle. *Nature* 172: 84–85, 1953.
16. Lorand, L. and C. Moos. Pyruvate phosphokinase system as a relaxing factor in glycerinated muscle. *Fed. Proc.* 15: 121, 1956.
17. Goodall, M. C. Carnosine phosphate as phosphate donor in muscular contraction. *Nature* 178: 539–540, 1956.
18. Lorand, L. Adenosine triphosphate-creatine transphorylase as relaxing factor of muscle. *Nature* 172: 1181, 1953.
19. Goodall, M. C. Auto-oscillations in extracted muscle fibre systems: creatine phosphate-adenosine triphosphate. *Nature* 177: 1238–1239, 1956.
20. Lorand, L. Auto-oscillations in extracted muscle fibre systems: pyruvate phosphokinase. *Nature* 178: 1471–1472, 1956.
21. Moos, C. and L. Lorand. Studies on the biochemistry of contraction and relaxation in glycerinated muscle. The effects of phosphoenolpyruvate. *Biochim. et biophys. acta.* 24: 461–474, 1957.
22. Goodall, M. C. and D. E. S. Brown. Relaxing mechanism in muscle fiber systems in relation to temperature and pressure. Unpublished data.
23. Marsland, D. A. The mechanisms of cell division; temperature-pressure experiments on the cleaving eggs of *Arbacia punctulata*. *J. Cell. & Comp. Physiol.* 36: 205–227, 1950.
24. Ebbecke, U. Wirkung allseitiger Kompression auf den Froschmuskel. *Pflüger's Arch. ges. Physiol.* 157: 79–116, 1914.
25. Brown, D. E. S. and D. J. Edwards. A contracture phenomenon in skeletal muscle. *Am. J. Physiol.* 101: 15–16, 1932.

26. Gasser, H. S. Contractures of skeletal muscle. *Physiol. Rev.* 10: 35–109, 1930.
27. Jöbsis, F. Reversible contractures of skeletal muscle produced by physiological salt solutions low in NaCl. *Am. J. Physiol.* 183: 631, 1955.
28. Jöbsis, F. and D. E. S. Brown. The chloride withdrawal contracture in skeletal muscle. In preparation.
29. Edwards, D. J. Personal communication, 1936.
30. Washington, M. A., M. F. Abrighi, S. F. Sheet and R. W. Ramsey. Q_{10} of the maximum tetanic tension developed by isolated muscle fibers of the frog. *Science* 121: 445–446, 1955.
31. Csapo, A. and M. C. Goodall. Excitability, length tension relation and kinetics of uterine muscle contraction in relation to hormonal status. *J. Physiol.* 126: 384–396, 1954.
32. Jöbsis, F. Tetanus tension in relation to temperature and pH. Doctoral thesis. In preparation.
33. Hajdu, S. and A. Szent-Gyorgyi. Action of DOC and serum on the frog heart. *Am. J. Physiol.* 168(1): 159–170, 1952.
34. Cattell, McK. and D. J. Edwards. Conditions modifying the influence of hydrostatic pressure on striated muscle, with special reference to the role of viscosity changes. *J. Cell. & Comp. Physiol.* 1: 11–36, 1932.
35. Brown, D. E. S. and D. W. Misch. Tension in auricular muscle in relation to temperature, pH and pressure. Unpublished data.
36. Hajdu, S. Observations on the temperature dependence of the tension developed by frog muscle. *Arch. Int. Physiol.* 61: 58–61, 1951.
37. Twente, J. A. The nature of treppe. Doctoral dissertation. University of Michigan, 1955.
38. Dawson, W. R. and G. A. Bartholomew, Jr. Cardiac and metabolic responses to temperature in *Dipsosauras dorsalis*. Unpublished data.
39. Edwards, D. J. and McK. Cattell. The action of compression on the contraction of heart muscle. *Am. J. Physiol.* 93: 90–96, 1930.
40. Cattell, McK. and D. J. Edwards. The influence of hydrostatic pressure on the contraction of cardiac muscle in relation to temperature. *Am. J. Physiol.* 93: 97–104, 1930.
41. Brown, D. E. S. The contraction of heart muscle in relation to temperature and pressure. In preparation.
42. Goodall, M. C. Unpublished, 1956.
43. Brown, D. E. S. The sequence of events in the contraction of heart muscle as revealed by abrupt compressions and decompressions. In preparation.
44. Brown, D. E. S. The effect of rapid compression upon events in the isometric contraction of skeletal muscle. *J. Cell. & Comp. Physiol.* 8: 141–157, 1936.
45. Brown, D. E. S. The effect of rapid changes in hydrostatic pressure upon the contraction of skeletal muscle. *J. Cell. & Comp. Physiol.* 4: 257–281, 1934.
46. Bishop, G. H. and A. I. Kendall, Action of formalin and histamine on tension and potential curves of a striated muscle, the retractor penis of the turtle. *Am. J. Physiol.* 88(1): 77–86, 1929.

Investigations 2, 8, 9, 17, 19, 22, 28, 35, 41, aided by a grant from the Muscular Dystrophy Association of America, Inc.

TEMPERATURE-PRESSURE STUDIES ON THE ROLE OF SOL-GEL REACTIONS IN CELL DIVISION[1]

Douglas Marsland, *Washington Square College, New York University, New York City*

Reversible sol-gel transformations have been observed in various cells for many years. It was not until 1926, however, starting with Mast's classical work on amoeboid movement (25), that the functional significance of these transformations began to be understood. Now, about thirty years later, there is considerable evidence which indicates that protoplasmic gel structures are potentially contractile. The gelational process seems to represent a mechanism which enables the cell to perform mechanical work. The purpose of this paper is to review the evidence, with special reference to the furrowing movements (cytokinesis) in animal cells. It seems probable that the development of mechanical energy by muscle tissues represents a related phenomenon, but the present discussion will be concerned primarily with contractile processes in the less highly specialized egg cell.

A priori, the development of contractility in an essentially fluid system, such as protoplasm, would seem to presuppose the formation of some kind of gel structure. If, as seems likely, the contractile force originates from the folding of elongate protein molecules or molecular aggregates, it is difficult to see how such a folding could be effective in performing work unless the extended protein units were interlinked, forming a continuous and fairly extensive system throughout the cell (5). Subsequent to folding, moreover, when the extended units have assumed a more globular form, the system can revert to the sol condition, merely by the loosening of the intermolecular bonds. The final dimensions, after folding in some gels, may represent a rather small fraction (about 10%) of the original volume of the gel as a whole. Thus the work performed during contraction may be considerable. Moreover, since the primary orientation of the elongate protein components may not be unidirectional, such systems need not display anisotropy, either in the expanded or contracted state.

Gel Structure in Relation to Pressure and Temperature. The early work of Brown (1) on Arbacia eggs and of Brown and Marsland (2) on two species of amoeba, showed that a drastic weakening and finally a

[1] Work supported by grant series CS07 from the National Cancer Institute, U. S. Public Health Service.

complete solation of the plasmagel structure of these cells occurred when they were exposed to increasingly high hydrostatic pressures in the range up to 8000 lb/in^2. This result was difficult to understand until Freundlich (4) published his analysis of gelational phenomena. Then it was realized that protoplasmic gel systems, of which quite a number have now been studied (21), are uniquely different from such more familiar types as gelatin or agar. Instead of solating when warmed, protoplasmic gel structures become more firmly set; and when higher pressures are applied, cellular gel structures are weakened and eventually solated—which is just opposite to the behavior of the gelatin system (23). In short, since protoplasmic gelations are endothermic and since a volume increase (ΔV) is involved, the sol-gel equilibrium is shifted toward the right by increasing temperature, and toward the left by increasing pressure. Thus both temperature and pressure have provided useful tools not only for analyzing the functional significance of intracellular gelations, but also for study in the metabolic processes which supply energy to these reactions. The molecular theory of volume changes accompanying various biological processes is discussed at some length in Johnson, Eyring and Polissar (7).

Special Techniques and Apparatus. Direct observation of the living specimens during exposure to high pressure is most essential. Characteristically the effects of pressure are rapidly and completely reversible and in several instances earlier workers obtained erroneously negative results simply because their observations were delayed until after decompression, following the removal of the material from the pressure chamber. Consequently progress in the field was greatly facilitated by the development of a microscope-pressure chamber in which small organisms and individual cells may be subjected to pressures up to 15,000 lb/in.^{2} (22, 21). Also the development of the pressure-centrifuge (1) and of an adaptable temperature control housing (21) were equally important. This equipment, in fact, has made it possible to obtain centrifugal measurements of the gelational state of the cytoplasmic components of different cells under systematically varied conditions of temperature and pressure throughout the physiological ranges, and to relate these gelational measurements to the observed changes in the activity and behavior of the cells.

Measurements of the relative gel strength have now been made on a number of different cells, including two species of amoeba (2) two species of *Arbacia* (1, 17, 18), and *Elodea canadensis* (19); and the consistent pattern of all these data tends to justify the assumptions upon which the method is based.

CYTOKINESIS

Background. Normal cell division unquestionably represents a complex series of events and processes. Here, however, we shall deal mainly with the furrowing process, by which the animal type of cell divides itself into two daughter cells. The evidence to be reviewed represents a very thorough testing of the cortical gel contraction theory of cytokinesis. This theory, as formulated by Marsland (21), postulates that the furrowing potency in animal cells depends upon the structural state and hence the contractile capacity of a strongly gelated cortical layer of cytoplasm, especially in the furrowing region. Thus the basic mechanisms for the development of mechanical energy in cytokinesis and in amoeboid movement are thought to be essentially the same.

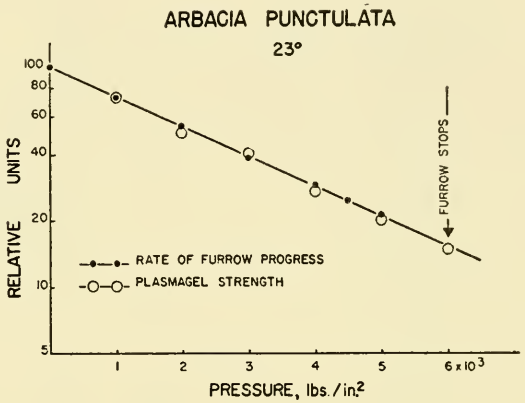


Fig. 1. Plasmagel strength and rate of progress of furrows in relation to high pressure. Data from Marsland, 1938.

The existence of a cortical plasmagel layer in an egg-cell (*Arbacia punctulata*) was first demonstrated clearly by the experiments of Dugald Brown in 1934 (1). These experiments showed that the cortically embedded pigment bodies (red chromatophores) are considerably more resistant to centrifugal displacement than those in the deeply-lying (medullary) cytoplasm of the egg. And in addition Brown showed that this well-defined plasmagel layer, like that of the Amoeba, is susceptible to solation under pressure; and that the plasmagel layer of the egg becomes very much more firmly gelled prior to and during the furrowing period.

Pressure Effects in Dividing Eggs. The main effects of pressure on the gel structure and furrowing performance of *Arbacia* eggs during first cleavage are summarized in figure 1. Here it may be seen *1*) that an exponential weakening of the cortical gel structure occurs as the pressure increases; *2*) that the progress of the furrow as it impinges on the spindle

axis becomes proportionally slower as this weakening of the gel system progresses; and *3)* that furrowing is aborted completely at 6,000 lb/in.^{2} (at 23°C), when the weakening of the plasmagel system has reached a certain critical level.

Although the pressure block to cleavage was first observed in the egg of *Arbacia punctulata,* many other eggs, derived from animals in five different phyla, have now been studied (27). The minimum pressure required to block the furrowing reaction shows some variation, to be sure,

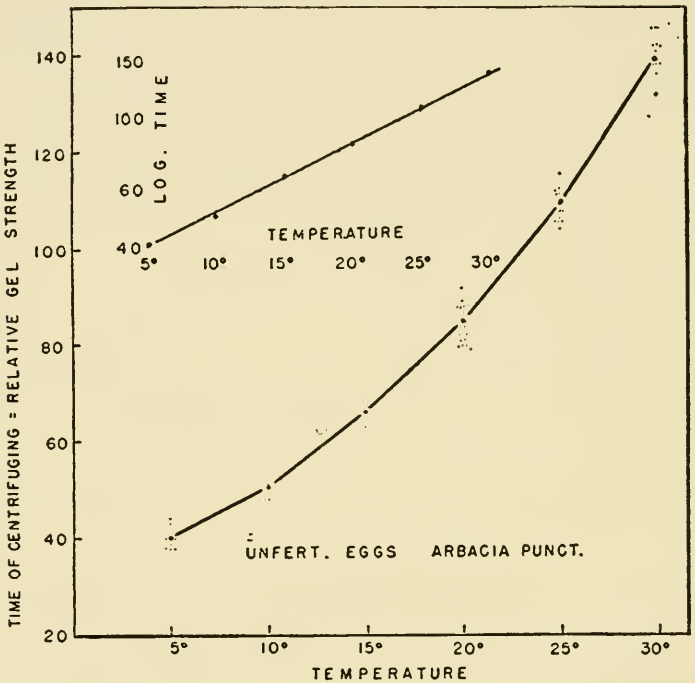


Fig. 2. Plasmagel strength in relation to temperature. Data from Marsland, 1950.

but in each case there is a critical blocking pressure and usually this falls in the range of 4,000–6,000 lb/in.^{2} (at 20°–25°C).

At pressures below 6,000 lb/in.^{2}, the furrow (of *Arbacia)* does not recede, but the rate at which it cuts through the egg is definitely retarded. At the 4,000-lb. level, for example, it takes 9 minutes for the furrow to pass from the equator to the spindle center, instead of the normal atmospheric time of 3 minutes (at 23°C). Thus the curve obtained by plotting the log of this retardation as a function of pressure gives an excellent fit when superimposed upon a similar plot of the cortical gel strength in relation to pressure, measured at the time when the furrows are about to form (fig. 1).

Temperature Effects. The data plotted in figures 2 and 3 show that the cortical plasmagel of the egg cell may be characterized as the type of system in which the structural strength of the gel net-work increases exponentially as the temperature is raised. This applies not only to the relatively weak gel system of the unfertilized egg (fig. 2) but also to the strongly fortified gel of the furrowing egg (fig. 3), where much higher centrifugal forces were required to achieve a displacement of the embedded chromatophores even though fairly high pressures were employed to bring the gel strength down into a measurable range.

Correlated apparently with this action of temperature in fortifying the structure of the plasmagel system, the furrowing strength of the egg be-

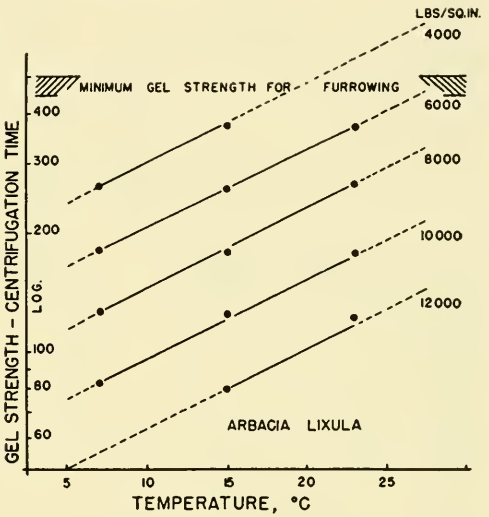


Fig. 3. Plasmagel strength in relation to temperature at various pressures. Measurements made on cleaving eggs of *Arbacia lixula*. Data from Marsland and Landau, 1954.

comes progressively greater at higher temperatures—as may be seen in figure 4. Thus with each increment of 5°C, the magnitude of the pressure required to block the furrows becomes greater—by about 1,000 lb/in.^{2} for the eggs of *Echinarachnius* and *Arbacia*—and by about 500 lb/in.^{2} for the Amphibian and Annelid eggs. Moreover, the correlation between gel structure and furrowing strength becomes even plainer in light of the data shown in figure 5. This family of curves shows that a furrowing block occurs whenever the structural strength of the cortical gel system falls to a certain critical level. At higher temperatures the pressures required to produce this critical weakening are higher, and at lower temperatures they are lower; but in each case the cell is rendered incapable of performing the work of cleavage by any combination of temperature and pressure treatments that weakens the gel structure to approximately the same degree.

General Interpretation. The temperature-pressure data (figs. 2–5) indicate that there is a quantitative relationship between the gelational state of cortical plasmagel layer in the equatorial region of the dividing cell and the strength of the furrowing reaction. Recent workers, indeed,

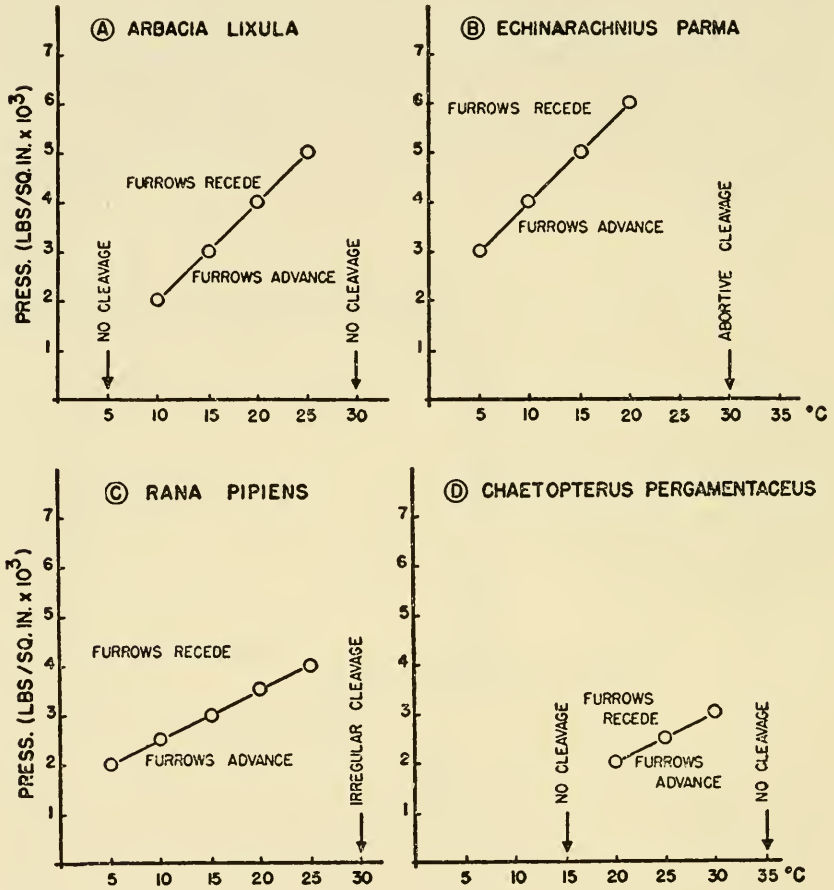


Fig. 4. Temperature variations in the minimum pressure required to block the first cleavage furrow in several different eggs. Data from Marsland and Landau, 1954.

are generally agreed that a decisive role in cytokinesis is played by the cortical plasmagel layer of the cytoplasm. However, there is no agreement as to the precise mechanism of the furrowing process. In fact, two opposite viewpoints are currently held: *first* that the cell membrane is pushed down into the developing furrow by a process of 'growth' (30, 3) or of 'expansion' (31, 26) occurring in the cortical protoplasm; and *second*,

that the cell membrane is pulled inward into the furrow by a contraction of the subjacent plasmagel in the equatorial region (12–14, 17, 20, 21). Here, however, only the second viewpoint will be presented.

As a prelude to division, before the cell elongates or displays any definitive furrow, a structural gradient must develop in the plasmagel system, which initially seems to be strongly and uniformly set throughout. Cytokinesis is initiated, apparently, by a localized solation which weakens the gel structure at each pole, as is indicated in figure 6*B*. Such a polar weakening is revealed by the experiments of Edward Chambers (personal communication) in which it was found that early telophase eggs exposed to hypotonic swelling always burst at the poles. Also it is found that

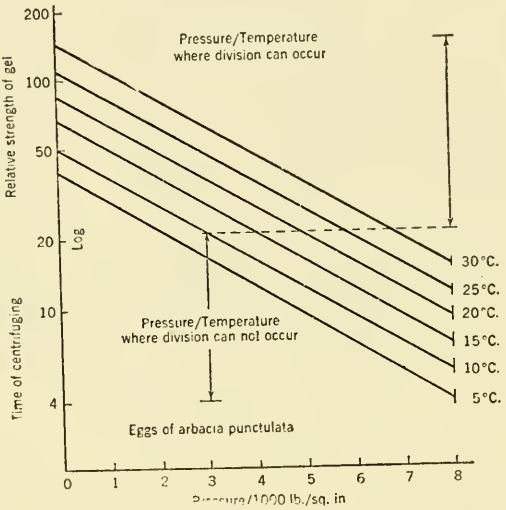


FIG. 5. Effects of temperature and pressure on the structural state of the plasmagel layer of the *Arbacia* egg in relation to cleavage capacity. Note that furrowing is aborted whenever the plasmagel strength falls below a critical level, as a result of the various temperature-pressure combinations. The centrifuge times are given directly, in seconds. Data from Marsland, 1950.

the pigment bodies at the poles, compared to those in the equatorial zone, are more readily displaced when early telophase eggs are subjected to high force pressure-centrifugation. This polar weakening, apparently, permits the more strongly gelled band-like portion of the plasmagel system in the broad central zone to contract, forcibly bulging the cell out at the poles as the cell undergoes elongation, and stretching the cell membrane in the polar regions. Such a polar stretching seems quite plain in the dividing neuroblasts depicted by Roberts (28), although in this case one pole appears to display solation slightly before the other and Roberts attributes the polar bulging to an active expansion rather than to a passive stretching. At present, however, there does not seem to be any decisive evidence which makes it necessary to assume that an active process of expansion provides energy for furrowing; and in the absence

of such evidence it is advisable to retain the simpler theory, namely, that the cell in furrowing, as it does in amoeboid movement, derives mechanical energy entirely from the contractility of the plasmagel system.

Following the contraction of the broad central zone, which causes the cell to elongate, the contracting part of the plasmagel becomes more narrowly restricted to the equator (fig. 6C). This leads to the appearance of a definitive furrow, as is likewise postulated in the surface expansion theory. Thus the area of passive surface stretching also becomes greater, so that now the sides of the prospective blastomeres near and even in the developing furrow become involved. Moreover, as the furrow

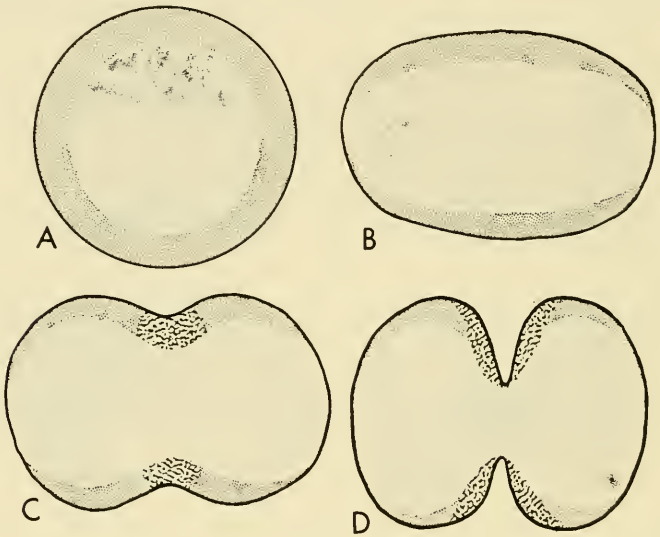


Fig. 6. Concept of the gel contraction mechanism of cytokinesis. Light shading indicates sol; medium shading = gel, contracting moderately; dark shading = gel, contracting strongly. A—anaphase; B. C and D—early, middle and late telophase.

deepens, new plasmagel is drawn into an operative position along the walls of the furrow. Thus in a well-developed furrow there are two fairly broad 'annuli' of newly mobilized plasmagel constituting the walls of the deepening furrow. This newly mobilized plasmagel can now contract and complete the work of cleavage. Meanwhile, the plasmagel at the very trough of the furrow, having expended its contractile energy, is free to undergo solation. This permits the cell membrane to fuse at the division axis, thus finally separating the daughter blastomeres (24).

KINETICS OF PROTOPLASMIC CONTRACTILITY

Earlier Evidence. Since protoplasmic gelations are endothermic, we must look for a basic metabolic pattern by which the cell provides energy

and determines when and where its essential gel structures shall be formed. Apparently the metabolic energy which the cell diverts into the building of its gel structures finally appears in the form of mechanical work during the contraction phase of the sol-gel cycle.

The importance of adenosine triphosphate (ATP) as an energy source in many tissues naturally suggested that this important metabolite might contribute energy to the sol-gel cycle in cells generally. Indeed, considerable evidence in this regard has begun to come from several directions. Runnstrom (29) showed that egg cells immersed in ATP solution became more resistant to hypotonic cytolysis, which seemed to be related to a gelling effect upon the cytoplasm. Kriszat (8, 9) found that ATP distinctly modifies the movements of *Amoeba proteus* (Chaos chaos). Loewy (16) extracted an actomyosin type of protein from the amoeboid slime mold, *Pelomyxa*, and showed that this preparation displayed considerable changes in its gelational structure in the presence of ATP and related compounds. H. H. Weber demonstrated that glycerol extracted fibroblasts underwent a quick and forceful contraction of their elongate pseudopodia when treated with ATP solutions and that this remarkable contraction could be stopped quickly and reversibly when the cells were treated with mersalyl acid (salyrgan), a compound that inhibits the hydrolytic splitting of the high energy bonds of ATP (32). And finally, Hoffman-Berling (6) found that fibroblasts killed and glycerol-extracted just at the beginning of telophase, when shallow cleavage furrows first appeared, showed a remarkable deepening of the furrows—virtually to the point of complete cleavage—when appropriate concentrations of ATP were added to the immersion medium. Accordingly, adenosine triphosphate was chosen as the first metabolite to be investigated in relation to the temperature-pressure parameters of the plasmagel system; and mersalyl acid (salyrgan) was used as an inhibitor of the ATP system.

ATP Effects on Furrowing Strength. Both *Arbacia* and *Chaetopterus* eggs displayed a distinct increase in the strength of the furrowing reaction as a result of adding ATP (0.0005 molal) to the sea water, starting approximately 25 minutes prior to the onset of first cleavage. This may be seen in figures 7 and 8 which show that the minimum pressures required to block the furrows are distinctly higher at each of the different temperatures. At atmospheric pressure also, the additional ATP enabled the eggs to complete their furrowing at temperatures which ordinarily are too low to allow for successful cleavage. Specifically for *Arbacia*, more than 90% of the ATP-treated eggs achieve successful furrowing at 9°C (compared to 10% for the untreated specimens); and for *Chaetopterus* at 17°C, 89% of the treated eggs went through (compared to 4% in the untreated specimens). In fact, to achieve an equivalent low temperature inhibition

of furrowing in the ATP-treated eggs, it was necessary to reduce the temperature by 2°, to 7° in *Arbacia* and to 15° in *Chaetopterus*.

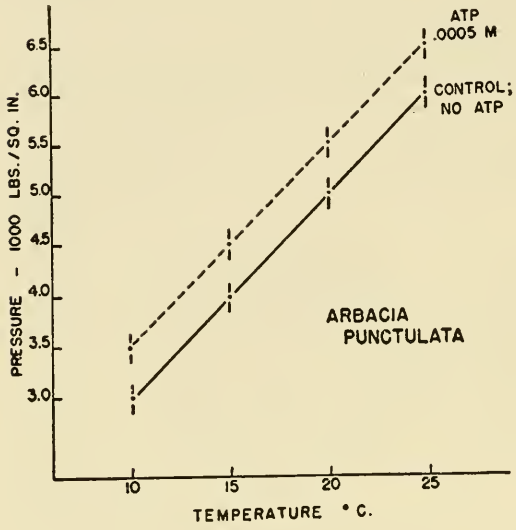


Fig. 7. ATP-induced increase in the strength of the furrowing reaction. Each point indicates the pressure level which is just sufficient to block the furrows at each given temperature. Data from Landau *et al.*, 1955.

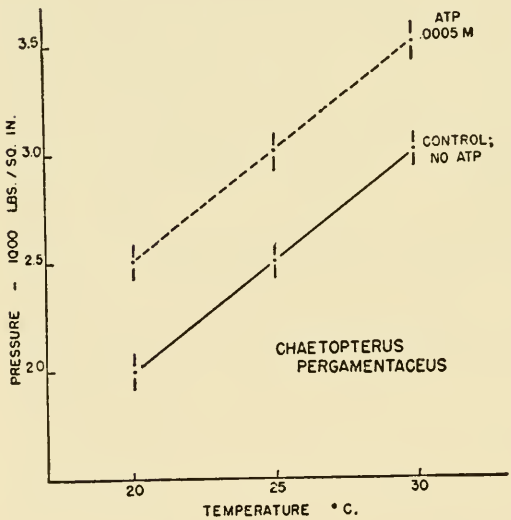


Fig. 8. Another example of the ATP-induced strengthening of the furrowing reaction. Points are same as in fig. 7. Data from Landau *et al.*, 1955.

ATP Effects on Gel Structure. The data presented in figures 9 and 10 provide further support for the view that the improved furrowing performance of the ATP-treated eggs results from a fortification of the structure of the plasmagel layer. However, further experiments should

be done. The present data were derived from the unfertilized eggs of the *Arbacia*, because centrifugal forces high enough to measure the exceedingly great gelational strength of the furrowing eggs were not available. Moreover, the concentration of ATP required to give a measurable effect on

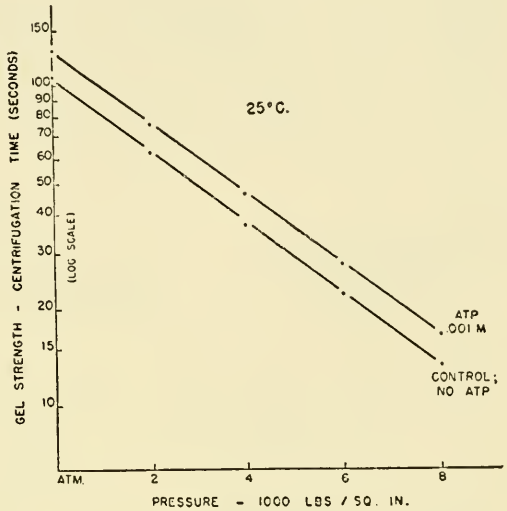


FIG. 9. ATP-induced fortification of the plasmagel structure. Centrifugal measurements and at *various pressures* on the unfertilized eggs of *Arbacia punctulata*. Data from Landau *et al.*, 1955.

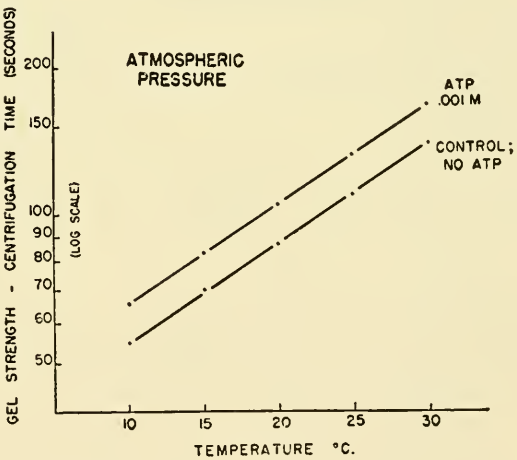


FIG. 10. ATP-induced strengthening of the plasmagel structure. Measurements at various temperatures on the unfertilized eggs of *Arbaci punctulata*. Data from Landau *et al.*, 1955.

the gel structure was twice that used in demonstrating the increased strength of the furrowing reaction. Virtually no effects were obtained when adenosine monophosphate, adenosine or inorganic phosphate was used in place of ATP (11).

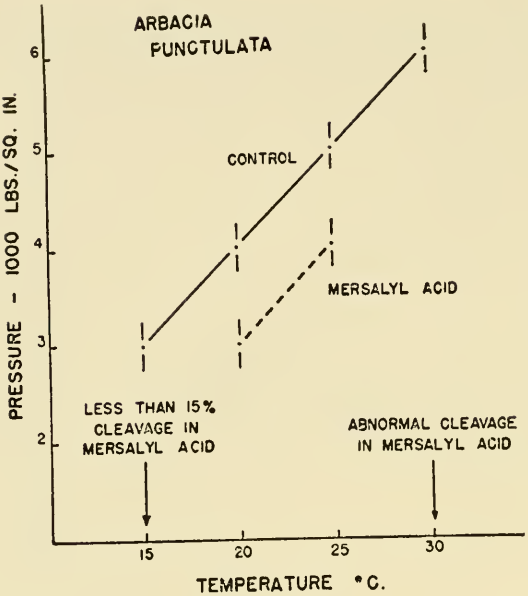


FIG. 11. Decreases in the strength of the furrowing reaction induced by mersalyl acid. Each point indicates the minimum pressure level required to block the first cleavage furrow. Data from Landau *et al.*, 1954.

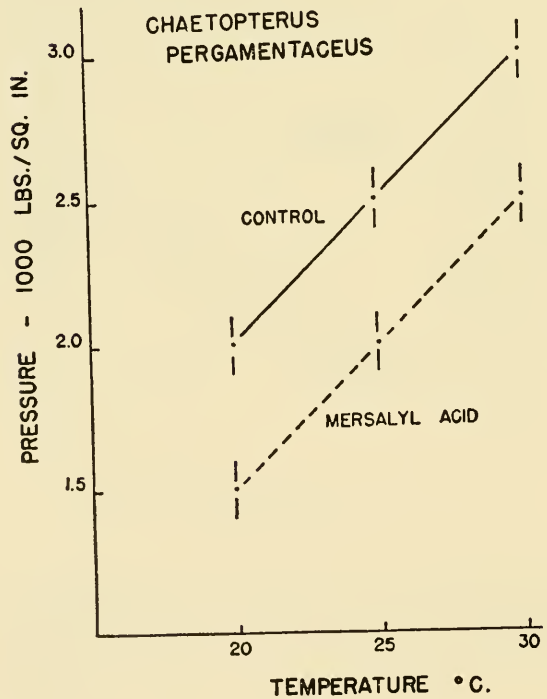


FIG. 12. Mersalyl acid-induced weakening of the furrowing reaction in another egg. Points as in fig. 11. Data from Landau *et al.*, 1954.

It is difficult, of course, to believe that ATP, a large and highly polar molecule, can readily penetrate into the cells in significant amounts, and it may not be necessary to postulate such penetration. Perhaps energy from ATP in the surrounding medium is made available at the cell surface, as indicated by the work of Lindberg (15).

Experiments With Mersalyl Acid (Salyrgan). The experiments of Weber and Portzehl (32) indicate that salyrgan strongly inhibits the hydrolytic splitting of the high energy phosphate bonds of ATP and consequently studies have been started on the effects of this compound upon the

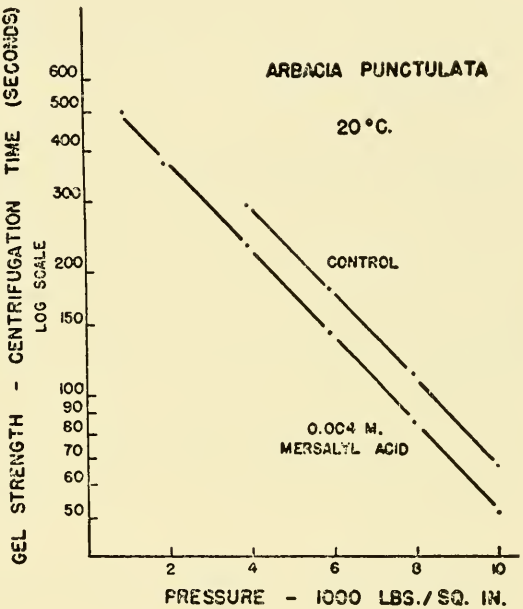


FIG. 13. Mersalyl acid-induced weakening of the plasmagel strength. Measurements made exactly 5 minutes prior to furrowing. At this time the gel strength reaches the very high cleavage maximum.

furrowing strength and gel structure of dividing eggs (*Arbacia* and *Chaetopterus*). The results so far likewise indicate that energy from the phosphate splitting can be utilized in building up the structure of the plasmagel system and finally serves to fortify the furrowing reaction. These results are shown in figures 11, 12 and 13. Here it may be seen that salyrgan added to the sea water (10 min. subsequent to fertilization) at a concentration (0.004 molal) which is not adequate to block the first cleavage, does nevertheless produce a distinct weakening of the furrowing reaction at each of the temperatures tested. Thus for both *Arbacia* and *Chaetopterus*, a lower (by 500 lb/in.^{2}) minimum pressure is required to block furrowing at each different temperature (figs. 11 and 12). This weakening of the furrowing strength is related apparently to a simultaneous weaken-

ing of the cortical gel structure (fig. 13). These measurements of the structural state of the gel system were made upon eggs which were about to divide (5 min, prior to furrowing). At this time the gelational state of the cortical cytoplasm had already risen sharply to its high maximum, and a centrifugal force of 25,000 × g was required to achieve a good displacement of the cortically embedded pigment bodies within a reasonable period of centrifugation.

GENERAL CONCLUSIONS

All in all, the various experiments here reported seem to provide substantial support for the hypothesis that gel structures formed by the cell, particularly the cortical plasmagel layer of the cytoplasm, are intrisically contractile and thus are instrumental in the performance of mechanical work. Metabolic energy, diverted into the endothermic gelation process, apparently, reappears in the form of mechanical work, the work of cytokinetic furrowing, when the fibrillar protein components of the gel structures undergo a forceful folding—thus reconverting the protein units into the mode globular form which is charcteristic of the sol condition.

Apparently the strength of the intermolecular linkages of the gel structure is a critical factor in determining the strength of the contractile force that it can develop. In any event, under widely varying experimental conditions and with several different kinds of cells it has been found that whenever the tensile properties of the plasmagel structure are weakened, the force of the mechanical movements of the cell is porportionately reduced. And conversely, all treatments that fortify the plasmagel structure have been found to give a corresponding increase in the mechanical performance.

Some evidence is now on hand which indicates that the high potential phosphate bonds of adenosine triphosphate may be utilized by the cell in deriving energy for the building of its gel structures. However, further studies are needed on the metabolic aspects of protoplasmic gelations.

SUMMARY

A survey of the evidence bearing on the mechanism of cytokinesis in marine eggs provides firm support for the hypothesis that cleavage results from the contraction of the strongly gelled cortical cytoplasm (plasmagel layer), first in the equatorial region and then in the walls of the deepening furrow. The strength of the furrowing reaction appears to be determined by the structural state of this plasmagel layer, as measured by the centrifugal method. Whenever the structural strength of the plasmagel is weakened to a critical degree, by high pressure or by low temperature, acting singly or in combination, furrowing is aborted and cleavage fails. Also, lesser de-

grees of weakening produce a proportionate retardation in the progress of the furrows.

Evidence is submitted which indicates that the splitting of the high potential phosphate bonds of adenosine triphosphate may contribute energy to the formation of the plasmagel structure—which is an endothermic process. In any event, the furrowing reaction and the plasmagel structure are both strengthened when additional ATP is provided via the surrounding sea water, and weakened when mersalyl acid (salyrgan), an inhibitor of ATP hydrolysis, is employed.

Generally speaking, the data indicate that the gelation process is causally related to the development of protoplasmic contractility and represents a mechanism by which the cell utilizes metabolic energy in the performance of mechanical work.

REFERENCES

1. Brown, D. E. S. The pressure coefficient of viscosity in the eggs of *Arbacia punctulata*. *J. Cell. & Comp. Physiol.* 5: 335–346, 1934.
2. Brown, D. E. S., and D. A. Marsland. The viscosity of *Amoeba* at high hydrostatic pressure. *J. Cell. & Comp. Physiol.* 8: 159–165, 1936.
3. Chambers, R. Structural and kinetic aspects of cell division. *J. Cell. & Comp. Physiol.* 12: 149–165, 1938.
4. Freundlich, H. Some recent work on gels. *J. Phys. Chem.* 41: 901–910, 1937.
5. Goldacre, R. J., and I. J. Lorch. Folding and unfolding of protein molecules in relation to cytoplasmic streaming, amoeboid movement, and osmotic work. *Nature* 166: 497–499, 1950.
6. Hoffman-Berling, H. Die Glycerin-wasserextrahierte Telophasezelle als Modell der Zytokinese. *Biochim. et biophys. acta* 15: 332–339, 1954.
7. Johnson, F. H., Eyring, H., and Polissar, M. J. *The Kinetic Basis of Molecular Biology*. New York: Wiley, 1954.
8. Kriszat, G. Die Wirkung von Adenosinetriphosphat auf Amoeben. Archiv fur Zoologi 1: 81–86, 1949.
9. Kriszat, G. Die Wirkung von Adenosinetriphosphat und Calcium auf Amoeben (*Chaos chaos*). Archiv fur Zoologi 2: 477–490, 1950.
10. Landau, J. V., D. A. Marsland, and A. M. Zimmerman. Kinetics of cell division: action of mersalyl acid on the pressure-temperature cleavage block in the eggs of *Arbacia* and *Chaetopterus*. *Anat. Rec.* 120: 789, 1954.
11. Landau, J. V., A. M. Zimmerman, and D. A. Marsland. The energetics of cell division; effects of adenosine triphosphate and related compounds on the furrowing capacity of marine eggs. *J. Cell. & Comp. Physiol.* 45: 340–359, 1955.
12. Lewis, W. H. On the role of a superficial plasmagel layer in division, locomotion and changes in form of cells. *Archiv. fur Exp. Zellforsch.* 22: 270, 1938.
13. Lewis, W. H. The relation of viscosity changes to amoeboid locomotion and cell division. *The Structure of Protoplasm*, William Seifriz, ed. Ames, Iowa: Iowa State College Press, 1942, pp. 163–197.
14. Lewis, W. H. Cell division with special reference to tissue culture. *Ann. New York Acad. Sc.* 51: 1287–1294, 1951.
15. Lindberg, O. On surface reactions in the sea urchin egg. *Exper. Cell Research* 1: 105–114, 1950.

16. Loewy, A. G. An actomyosin-like substance from the plasmodium of a Myxomycete. *J. Cell. & Comp. Physiol.* 40: 127–156, 1952.
17. Marsland, D. A. The effects of high hydrostatic pressure upon cell division in *Arbacia* eggs. *J. Cell. & Comp. Physiol.* 12: 57–70, 1938.
18. Marsland, D. A. The mechanism of cell division. Hydrostatic pressure effects upon dividing egg cells. *J. Cell. & Comp. Physiol.* 13: 15–22, 1939a.
19. Marsland, D. A. The mechanism of protoplasmic streaming. The effects of high hydrostatic pressure upon cyclosis in *Elodea canadensis*. *J. Cell & Comp. Physiol.* 13: 23–30, 1939b.
20. Marsland, D. A. Protoplasmic streaming in relation to gel structure in the cytoplasm. *The Structure of Protoplasm*, William Seifriz, ed., Ames, Iowa: Iowa State College Press, 1942, pp. 127–161.
21. Marsland, D. A. The mechanisms of cell division; temperature-pressure experiments on the cleaving eggs of *Arbacia punctulata*. *J. Cell. & Comp. Physiol.* 36: 205–227, 1950.
22. Marsland, D. A., and D. E. S. Brown. Amoeboid movement at high hydrostatic pressure. *J. Cell. & Comp. Physiol.* 8: 167–178, 1936.
23. Marsland, D. A., and D. E. S. Brown. The effects of pressure on sol-gel equilibria, with special reference to myosin and other protoplasmic gels. *J. Cell. & Comp. Physiol.* 20: 295–305, 1942.
24. Marsland, D. A., and J. V. Landau. The mechanisms of cell division; temperature-pressure experiments on the cortical gel system in various marine eggs. *J. Exper. Zool.* 125: 507–539, 1954.
25. Mast, S. O. Structure, movement, locomotion and stimulation in Amoeba. *J. Morphol.* 41: 347–425, 1926.
26. Mitchison, J. M. Cell membrane and cell division. *Symp. Soc. Exp. Biol.* 6: 105–127, 1952.
27. Pease, D. C. and D. A. Marsland. The cleavage of *Ascaris* eggs under exceptionally high pressure. *J. Cell. & Comp. Physiol.* 14: (suppl.); 1–2, 1939.
28. Roberts, H. S. The mechanism of cytokinesis in neuroblasts of *Chortophaga viridifasciata*. *Anat. Rec.* 120: 726–727, 1954.
29. Runnstrom, J. The mechanism of fertilization in metazoa. *Adv. Enzymol.* 9: 241–327, 1949.
30. Schechtman, A. M. Localized cortical growth as the immediate cause of cell division. *Science* 85: 222–223, 1937.
31. Swann, M. M. The nucleus in fertilization, mitosis and cell division. *Symp. Soc. Exp. Biol.* 6: 89–106, 1952.
32. Weber, H. H., and H. Portzehl. The transference of the muscle energy in the contraction cycle. *Progr. Biophys.* 4: 60–111, 1954.

SYNCHRONIZATION OF CELL DIVISION BY CHANGES IN TEMPERATURE

Victor G. Bruce, *Department of Biology, Princeton University, Princeton, New Jersey*

If bacteria, and some other microorganisms, are allowed to grow in liquid culture to saturation, they reach a stage where they are not growing and dividing but are in a resting stage in which they are still viable. They are quite uniform in shape and size, although they are generally smaller than the growing cells in a rapidly dividing culture. When resting cells are introduced into fresh media they immediately start synthesizing protoplasm but because of their uniformly small size they do not for some time divide and hence there is a lag in cell division. Since the cells start off more or less in the same stage of development, one might expect that they would divide somewhat synchronously. Ordinarily this is not the case and the absence of synchrony arises as a consequence of the large variability in the division times of individual bacteria. This variability has been measured for some bacteria (17) and is sufficiently large that synchronized bacteria may rapidly get out of synchrony due to this effect alone. Using the simplified assumptions that the distribution of division times is of the normal curve of error type, together with the assumption that the cells are all initially synchronized at the stage immediately following division, one can calculate the form of the resulting growth curve. If there is to be any detectible synchronization effect, the maximum slope of the growth curve, which reflects the rate at which the cells are dividing, should be at least two times the slope of the straight line growth curve representing random exponential growth. In order for this to be the case the standard deviation from the mean of the distribution of division times must be less than 20%. The variances observed (17) for the distribution of division times in several types of bacteria were always considerably more than this. Observations by some workers have shown however that in some cases there may be a slight amount of synchrony of cells coming out of the lag phase. Hegarty and Weeks (5) and Houtermans (7) have reported observations of effects in bacteria which may be interpreted in this way, and McClintock (16; under certain conditions not investigated in any detail, it was observed that *Neurospora* develops synchronously if transferred from old cultures to fresh media) has observed a similar effect in *Neurospora*. In these cases a temperature change was also made at the

time the old cells were transferred to new media, and this might have had an effect in inducing the synchronization.

Recently, a number of reports have appeared describing methods whereby the randomness of cell divisions in exponentially growing cultures may be partially eliminated and the cultures thus synchronized. Many of these have involved temperature changes (6, 8–13, 18, 19, 23), and the interest in the present discussion will center on those systems using temperature changes or cycling to effect synchronization. Physical (14, 21) and biochemical (1) methods have also been used to effect some degree of synchronization, and it has been observed that alternating cycles of light and dark (20) have an effect on the synchronization of some algae. Various investigations on the physiological (3, 10, 13), biochemical (1, 4, 12), and cytological (8, 11, 19) aspects of these synchronized systems have been reported.

The purpose of this paper is to discuss these synchronization systems particularly in connection with possible influences of temperature and sudden temperature changes on the cell cycle. We shall start by briefly describing a schematic model of the cell division cycle.

CELL DIVISION CYCLE

Recent views on the life history of the cell have been discussed by Mazia (15). It has been shown for several microorganisms that cell growth in terms of weight, volume or protein content is most rapid immediately following division and thereafter slows down and apparently completely stops sometime prior to the next subsequent division. The period during which there is little or no synthesis is evidently a period of reorganization during which the cell prepares to divide. Although there had previously been a tendency to think of division as being initiated when the cell reaches a certain critical size, there are objections to such a view and the evidence for the existence of a 'trigger' leading up to division is emphasized by Mazia (15). Thus, this period of no synthesis might be subdivided into two periods, a pre-division period and a period during which the cells are dividing. Generally, cells which are already dividing cannot be stopped from dividing, whereas if the trigger is not pulled various treatments will prevent cells from entering the division stage. Although characterization of the trigger remains an outstanding problem, this general description of the cell cycle seems an appropriate one. This description of the cell cycle is based largely on observations on protozoa such as *Amoeba*, with which Mazia and his coworkers have done most of their work, and *Tetrahymena*, which Zeuthen and others have investigated. Measurements have been made using single cells as well as synchronized cultures.

The description of the division cycle seems appropriate also for bacteria, although here of course the evidence is necessarily more inferential since single cell measurements cannot be made. Suggestive evidence that the principal growth takes place only during a part of the division cycle derives from the fact that turbidity changes in a synchronized culture of *Salmonella typhimurium* increase in a step-wise fashion (2) as shown in figure 1. Thus, if little or no growth takes place during a considerable part of the division cycle, then there will be no turbidity increase during

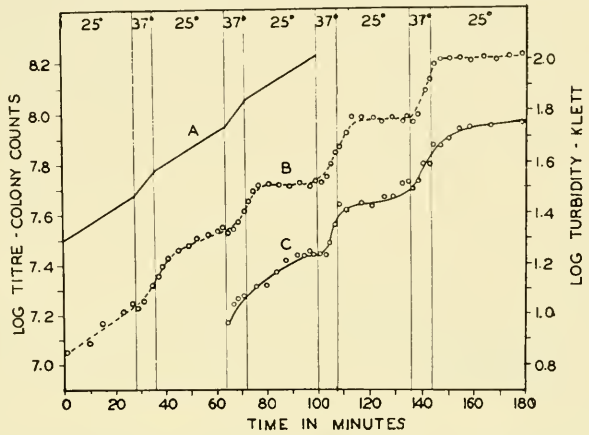


FIG. 1. Repeated temperature cycling experiment. An overnight 37° culture of *S. typhimurium* was diluted 1:100 in broth/5, grown with aeration from t = −90 min. to t = 0 min. at 37°, and then subjected to alternate cycles of 25° and 37°. The hot or cold broth, added to raise or lower the temperature of the culture, maintained the titer of the culture between 2 and 4 × 10^7 cells/ml A, ———, compound exponential growth curve constructed from the normal generation times of *S. typhimurium* in broth/5 at 25° and 37°; B, ———, turbidimetric reading in Klett colorimeter; C, ———, viable titer measured as colony count. Turbidity and colony counts have been corrected for culture dilutions.

the comparable part of the synchronized cycle. If this interpretation is valid, then one inference would be that in an exponentially growing culture only a part, perhaps only 30 to 50% of the cells, will be actively synthesizing protein. The addition to such an exponentially growing culture of the tryptophane analogue 5-methyl-tryptophane (5MT) might be expected to stop the growth of all cells which are still actively synthesizing protein and to allow those cells which are in the later phases of the division cycle to proceed toward division and perhaps to divide once in the presence of the 5MT. If, after roughly one half of the generation time has elapsed in the presence of the 5MT, an excess of tryptophane (T) is added, then one could expect a resumption of protein synthesis approaching asymptotically the exponential rate. One would also expect the colony count

growth curve to reflect this partial synchronization; however the form of the curve in the period when the 5MT is added and following the addition of T would depend on whether actual cell division is inhibited by 5MT. A necessary and sufficient requirement to make the above-described hypothesis agree with the experimental facts is that there should be some period during which the average cell size, as measured by

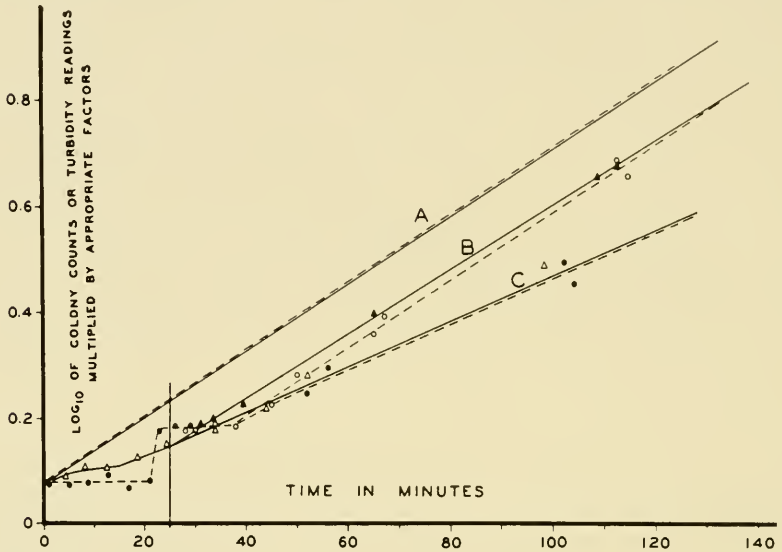


FIG. 2. Five methyl tryptophane (10^{-4}M) was added at t = 0 to an aliquot C of a culture A of *S. typhimurium* growing exponentially in M9 synthetic medium at 37°. At t = 25 min. excess tryptophane was added to an aliquot B from C. All cultures were left at 37°, and were kept between 3.5 and 7 × 10^7 cells/ml. by means of culture dilutions for which allowance has been made in the figure. Turbidity readings (solid line) and colony counts (broken line) for A were taken from t = −90 min to t = +200 min. and experimental points for curve A have been omitted from the figure.

——— Turbidity for C; ——— turbidity for B; -------- colony counts for C; -------- colony counts for B.

the turbidity and colony count curves, should be considerably smaller than the average cell size of an exponentially growing culture. This could also be expressed by the statement that there should be a period during which the colony counts increase more rapidly than the turbidity, followed by a period when the turbidity increases more rapidly than the colony counts. Eventually, of course, the colony count curve and the turbidity curve should again come together indicating that the average cell size is the same as before the addition of the 5MT. Experiments of this type were tried by O. Maaløe and the author, and figure 2 illustrates the re-

sults of one such experiment. A culture of *S. typhimurium* growing exponentially in synthetic medium at 37° has a generation time of about 45 minutes as determined from curve A in the figure. At zero time 10^{-4} M 5MT was added to a portion of this exponentially growing culture and at t = 25 minutes excess tryptophane was added to a portion of the culture containing 5MT. In this and in other similar experiments it was noted that 5MT initially inhibits cell division as well as growth but that subsequently the cells divide once in the presence of 5MT. The addition of excess tryptophane at a time when a large fraction of the cells are newly divided results in an increase in turbidity without a corresponding increase in colony counts. The jump in the colony count curve, occurring in the presence of 5MT, also occurs if the concentration of the 5MT is ten times higher. At the higher concentration of 5MT the turbidity increases even less than in the above-described experiment and the average cell size is correspondingly less.

There is thus evidence that in at least one respect the description of the cell division cycle is similar in bacteria and protozoa. The implication that there is a point during the later part of the cell cycle at which the cell enters more or less irreversibly into division, and that the processes leading up to this point are sensitive to being blocked by different methods, provides a natural explanation for some of the synchronization systems to be described.

SYSTEMS IN WHICH SYNCHRONIZATION HAS BEEN ACCOMPLISHED BY MEANS OF TEMPERATURE EFFECTS

Although some synchronization effects had been observed before 1953, it was not until 1953 and 1954 that workers began to develop synchronization methods as a means of invesigating the division cycle. Hotchkiss (6), Zeuthen and Scherbaum (18, 23), and James (9) independently decided on temperature changes as a means of inducing synchrony, and each used a somewhat different method. Maaløe and Lark (10, 13) were led to try temperature changes as a result of the work of Hotchkiss, and of Zeuthen and Scherbaum, and they also independently developed still a different method. Subsequently, Szybalski and Hunter-Szybalska (19) used a method very similar to that used by Hotchkiss.

Lark and Maaløe (10, 13) have investigated the effect of single and of multiple temperature shifts on the growth of cultures of *S. typhimurium*. They find that a culture growing exponentially at 37° will continue growing exponentially at a slower rate if the temperature of the culture is reduced to 25°. However, if a culture is growing exponentially at 25° and the temperature is raised to 37° the culture continues growing at the 25° rate for about 20 minutes, following which the growth rate is for a short time

faster than that found normally at 37°. The culture eventually grows exponentially at the normal 37° rate and the extrapolated exponential growth curves meet at the time where the temperature shift occurred. Thus, a single temperature shift in the *S. typhimurium* system does result in a small amount of synchronization. The characteristic behavior in this experiment is reproducible only if the culture has grown exponentially at 25° for several generations. It was also shown (10) that a 37° culture just entering the exponential phase, and exposed for a limited time to 25° after which it was returned to 37°, shows a more pronounced synchronization effect than is observed in the single temperature transfer experiment. The best synchronization effect resulting from the two temperature shifs occurs if the culture is exposed for about 30 minutes to the lower temperature, and it is probably significant that this time is less than, but of the order of magnitude of, the 45-minute generation time at 25°. Nevertheless, even with two temperature transfers, only a part of the cell population is synchronized as evidenced by approximately a 50% jump in colony counts occurring 5 to 10 minutes after the second temperature transfer. The most complete synchronization is achieved by means of repeated temperature transfers between 25° and 37°. The precise cycling conditions are different for dilute cultures and for cultures at densities of the order of 3 to 6 $\times$ 10^7 cells per ml, and synchronization has not been effected at densities higher than this. To synchronize cultures containing 3 to 6 $\times$ 10^7 cells per ml the following procedure is used. An overnight 37° broth culture is diluted 100-fold in broth and aerated at 37° for 75 minutes at which time it is just coming into the exponential phase of growth. The temperature is then alternately lowered to 25° for 28 minutes and raised to 37° again for 8 minutes. The temperature changes are made suddenly by diluting with hot or cold broth in such a way that the density of the culture remains within the limits given above. This temperature cycling is continued for four, five or six cycles and usually several cycles of synchronized growth are obtained in this way. If a culture is so synchronized and then left at 37° the growth rapidly becomes exponential, suggesting that the distribution of division times is very broad.

Hotchkiss (6) obtained synchronously dividing cultures of *Pneumococcus* by reducing the temperature of a culture growing in the late exponential phase in complex media from 37° to 25° for 15 minutes and then raising the temperature to 37° again. It was shown by means of viable counts that following the second temperature transfer two or three cycles of synchronous growth ensued with a gradual loss of synchrony. A cyclical fluctuation in the susceptibility to transformation also resulted, although not with quite the same period of the synchronous growth curve.

Scherbaum and Zeuthen (18, 23) induced synchronous division in the protozoon *Tetrahymena pyriformis* by alternately subjecting an exponentially growing culture to 30-minute periods at the optimal temperature of 29° and 30 minutes at the sub-lethal temperature of 33°. Treatment for 6 to 10 hours with this cycling causes the cells to grow bigger without dividing, and if they are subsequently returned to a lower temperature (24°), there follow successive peaks of division activity, about 1½ hours apart, separated by periods in which no divisions occur. In some of their early experiments these workers investigated the effect of temporarily lowering the temperature of a culture growing exponentially at optimal temperature. A synchronous burst of divisions (25–30% of the population) occurred after the temperature was raised. However, they were unable to improve the synchrony by repeating the temperature changes between the low (7°) and the optimal temperature.

Szybalski and Hunter-Szybalska (19), working with *Bacillus megaterium*, used a method very similar to that used by Hotchkiss in order to obtain synchronous divisions of this bacteria. The temperature of a culture growing exponentially at 34° was lowered to 15° for 30 minutes after which it was again raised to 34°. Two or three cycles of synchronous divisions occurred after the second temperature transfer, with a progressive loss of synchrony.

The last example to be cited here in which temperature changes have been used to synchronize divisions is the system described by James (9) for *Amoeba proteus*. In this system a number of dividing amoebae are manually selected and subjected to a 24-hour temperature cycle of 12 hours at 26°, and 12 hours at 18°. The dividing amoebae are started off at the beginning of the warm period and divide again at the beginning of the next warm period 24 hours later. The amoebae divide over a spread of time equal to about 5% of the total generation time and after five or six divisions begin to get out of synchrony again. This method of course combines physical techniques with temperature changes and thus is somewhat different from the previous systems.

DISCUSSION

The several systems which have been briefly described showing how temperature changes can effect synchronization of cell division raise the question of whether a common mechanism is operating and if so what is the nature of the temperature effect resulting in the synchronization. The main generalization that can be made about the effect of temperature on microbial growth is the accelerating of the division rate, with a Q_{10} of approximately 2 up to an optimum temperature, and with some falling off of the rate at higher temperatures. Speculations have linked this

temperature influence to the effect of temperature on a single enzymatic reaction, postulating the falling off at higher temperatures as being due to thermal denaturation. It may be that in some cases the temperature dependence of growth rate does correlate with a single reaction but this is probably not generally the case. The effect of sudden temperature changes on the metabolism or growth has been investigated hardly at all and in considering the physiological effects one should bear in mind the magnitude of the temperature changes with respect to optimal temperature and normal temperature range. Although there is outwardly considerable difference between the synchronization systems, it does seem that there may be a qualitatively similar effect of temporary exposure to a non-optimal temperature on a particular stage of the division cycle.

Hotchkiss (6) in interpreting the synchronization of *Pneumococcus* expressed the idea that the sudden cooling, to a temperature well below the optimum, of an exponentially growing culture might selectively slow down, or even stop, the metabolism of that fraction of the cells which had not passed a definite stage of the division cycle thus giving an opportunity for different cells to come into phase with each other. The later demonstration by Szybalski and Hunter-Szybalska (19) that an almost similar method will work with *B. megaterium* shows that the mechanism is not specific to a single organism. Furthermore, similar though smaller effects occur if *S. typhimurium* or *Tetrahymena* are subjected to a similar type of temperature treatment. Nevertheless, it should not be concluded that this represents a universal response of microorganisms to this type of temperature treatment. Experiments of this type by the author, using various strains of *E. coli*, failed to disclose any synchronization. Different types of temperature treatment can be effective in accomplishing the same net end result, e.g. selectively blocking a specific step in the division cycle. This is nicely illustrated by the experiments on *Tetrahymena* which involved a relatively complex temperature pretreatment in which the temperature was raised from the optimal level. Zeuthen (22) expressed the opinion that certain fractions of the cell population were differently affected by raising the temperature to the sublethal level. Dividing cells continue division and growth, cells hit between divisions continue synthesis; however cells hit in some stage just prior to division continue growth but do not for a time enter division.

Zeuthen's suggestion that the step which is blocked is one just before the cells enter into division is consistent with the results obtained with *Pneumococcus* inasmuch as the first synchronized burst of divisions in the *Pneumococcus* system occurs a few minutes after returning the culture to 37° from the cold. With *B. megaterium*, on the other hand, there is a lag following return of the culture to optimal temperature from the

cold, suggesting that the step in the cycle which is blocked occurs soon after division.

The results obtained with *S. typhimurium* and *Amoeba* suggest that the blocking effects of temperature changes is less complete and that repeated temperature changes are necessary for synchronization. In the case of *S. typhimurium* the step which is affected probably occurs fairly early in the division cycle. In the repeated temperature shift experiment the bursts of cell divisions occur within an 8- to 10-minute interval and this is explained as arising from individual differences between the cells. If the temperature cycling causes an accumulation of cells in a phase immediately following division, and if, when the cells get out of this phase, they take varying lengths of time to complete division, there will be some spread in the time interval during which the cells divide.

Probably there is more than one point in the division cycle at which the cells can be blocked, even by temperature changes alone, and it would be pure speculation to discuss the blocking mechanism in any more detail. It appears however that the mechanism whereby synchronization is effected is based on qualitative effects of the temperature changes on the metabolism of the cell, such as might be expected to occur if there were large differences in the temperature coefficients of different phases of the cell cycle.

SUMMARY

Recent work on the synchronization of the division cycle of bacteria and protozoa is reviewed and those systems in which synchronization has been effected by means of temperature changes are discussed in some detail. Recent ideas of the division cycle of the cell are discussed and, in particular, attention is drawn to the probable existence of one or more critical steps in the cycle which are particularly sensitive to being blocked. The effects of temperature changes in inducing synchronization are considered as probably arising from the prevention of the completion of a specific step in the cycle without affecting cells which have completed this step.

REFERENCES

1. Barner, H. D. and S. S. Cohen. Synchronization of a thymineless mutant of *Escherichia coli*. *J. Bact.* 72: 115, 1956.
2. Bruce, V. G., K. G. Lark and O. Maaløe. Turbidimetric measurement on synchronized *Salmonella typhimurium* cultures. *Nature* 176: 563, 1955.
3. Bruce, V. G. and O. Maaløe. The ultraviolet inactivation of synchronously dividing *Salmonella typhimurium*. Post irradiation effects on viability. *Biochim. et Biophys. acta* 21: 227, 1956.
4. Falcone, G. and W. Szybalski. Biochemical studies on the induction of synchronized cell division. *Exper. Cell. Res.* 11: 486, 1956.

5. Hegarty, C. P. and O. B. Weeks. Sensitivity of *Escherichia coli* to cold shock during the logarithmic growth phase. *J. Bact.* 39: 475, 1940.
6. Hotchkiss, R. D. Cyclical behaviour in pneumococcal growth and transformability occasioned by environmental changes. *Proc. Nat. Acad. Sci.* 40: 49, 1954.
7. Houtermans, T. Uber den Einfluss des Wachstumszustandes eines Bakteriums auf seine Strahlenempfindlichkeit. *Zeit fur Naturforshg.* 8b: 767, 1953.
8. Hunter-Szybalska, M. E., W. Szybalski and E. D. Delamater. *J. Bact.* 71: 17, 1956.
9. James, T. W. Ph.D. Thesis. Berkeley, Calif.: University of California, 1953.
10. Lark, K. G. and O. Maaløe, The induction of cellular and nuclear division in *Salmonella typhimurium* by means of temperature shifts. *Biochem. et biophys. acta* 15: 345, 1954.
11. Lark, K. G., O. Maaløe and O. Rostock. Cytological studies of nuclear division in *Salmonella typhimurium*. *J. Gen. Microbiol.* 13: 318, 1955.
12. Lark, K. G. and O. Maaløe. Nucleic acid synthesis and the division cycle of *Salmonella typhimurium*. *Biochim. et biophys. acta* 21: 448, 1956.
13. Maaløe, O. and K. G. Lark. A study of bacterial populations in which nuclear and cellular divisions are induced by means of temperature shifts. In: *Recent Developments in Cell Physiology. Symp. Colston Res. Soc.* 7: 159, 1954.
14. Maruyama, Y. and T. Yanagita. Physical methods for obtaining synchronous cultures of *Escherichia coli*. *J. Bact.* 71: 542, 1956.
15. Mazia, D. The life history of the cell. *Am. Sci.* 44: 1, 1956.
16. McClintock, B. Personal communication.
17. Powell, E. O. Some features of the generation times of individual bacteria. *Biometrika* 42: 16, 1955.
18. Scherbaum, O. and E. Zeuthen. Induction of synchronized cell division in mass cultures of *Tetrahymena pyriformis*. *Exper. Cell Res.* 6: 221, 1954.
19. Szybalski, W. and M. E. Hunter-Szybalska. Synchronization of nuclear and cellular division in *Bacillus megaterium*. *Bact. Proc.*, p. 36, 1955.
20. Tamiya, H., T. Iwamura, K. Shibata, E. Hase and T. Nihei. Correlation between photosynthesis and light-independent metabolism in the growth of *Chlorella*. *Biochim. et biophys. acta* 12: 23, 1953.
21. Yanagita, T. Single step growth method for *Escherichia coli* B. (Read at the 54th General Meeting of the Society of American Bacteriologists, May, 1954.)
22. Zeuthen, E. 1954. Personal communication.
23. Zeuthen, E. and O. Scherbaum. Synchronous divisions in mass cultures of the ciliate protozoon *Tetrahymena pyriformis*, as induced by temperature changes. In: *Recent Developments of Cell Physiology. Symp. Colston Res. Soc.* 7: 141, 1954.

TEMPERATURE ALTERATIONS OF RESPONSE OF CELL DIVISION TO URETHAN

Ivor Cornman, *Hazleton Laboratories, Falls Church, Virginia*

The precise timing of echinoderm egg cleavage makes these eggs conveniently useful in rate studies. But, being whole cells—in fact, whole eggs—they are complex, and this complexity obscures the view when one is looking for the basic mechanisms. A great many things affect the division of these eggs (cf. Harvey, 4), and it is inconceivable that all influences retarding cleavage act in the same way. The egg obliges with some visual clues, however. Within its limited repertoire there are distinctive patterns of response. The pattern induced by carbamates is distinctive, although not unique. It does permit one to say with some conviction that most of the simpler carbamates act in the same way, despite considerable variation in molecular build (2).

One recognizable part of the pattern is the extent to which cleavage can be slowed without destroying the egg. Figure 1 shows this in the *Echinarachnius* egg. First cleavage was at about 110 minutes, and second cleavage at 170 minutes at 20.2 ± 0.1°C. Eggs exposed to 2.2 mM reached 50% first cleavage at four hours and did not complete the first cleavage until about six hours after fertilization. Yet they persisted and formed blastulae the next day. Half the concentration produced a two-minute delay of first cleavage; twice as much blocked cytoplasmic division completely in some of the eggs, but some went on to produce irregular ciliated cell clumps. This is quite in contrast to a mitotic poison like colchicine or podophyllin. One can establish a threshold dose of these substances that will produce a slight delay and permit continued cleavage, but any slight increase in concentration blocks division completely and eventually destroys the egg. One cannot get a prolonged retardation with these metaphase-blocking poisons without completely disrupting ontogenesis.

One aspect of the urethan curve in figure 1 is at once confusing and revealing. The cleavage percentage reached 60% at 154 minutes, then dropped to 22%. This is another phenomenon typical of the carbamates—and again, not restricted to them. At the higher doses that still permit cleavage, there is an initial spurious division. To all appearances the eggs have divided, and one must count them as two-cell when following a population of living eggs. Subsequently these supposedly complete furrows widen into shallow grooves and eventually disappear, leaving a binucleate

egg. Then the delayed first cleavage and the second cleavage progress almost simultaneously. There is more than coincidence in this. It reflects a fundamental relationship between the achromatic figure and cytoplasmic cleavage. One can observe in these eggs, coincident with the postponement of cleavage, a mitotic figure smaller than normal. It is reasonable to suppose that during the first mitotic cycle the division figure was too weak to divide the cytoplasm completely. The nuclear cycle continued, however,

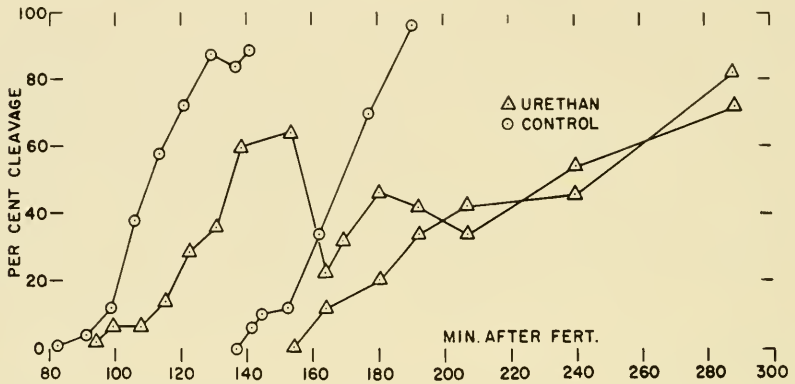


FIG. 1. Retardation and regression of *Echinarachnius* egg: cleavage in urethan. Progress of cleavage with time, observed in living *Echinarachnius* eggs. Control cleavage reached 50% at 110 minutes and neared completion at 130 minutes. Exposure to 22 mM urethan resulted in temporary furrowing at 140 and 180 minutes. Then first and second cleavage proceeded simultaneously as a result of multiple cleavage.

so that two figures formed and divided the egg simultaneously into four cells. As the dose is increased, this abortive phase is also suppressed and cleavage comes much later. There is also some abortion of the second attempt at cleavage, represented by the hump at 180 minutes.

TABLE 1. VARIANTS OF ESTERS AND N-SUBSTITUTED CARBAMATES*

$HNHCOOCH_3$—methyl carbamate
$HNHCOOCH_2CH_2CH_3$—propyl carbamate
$HNHCOOCH_2CH_3$—ethyl carbamate
$C_6H_5NHCOOCH_2CH_3$—ethyl-N-phenyl carbamate
$(CH_2CH_3)_2NCOOCH_2CH_3$—ethyl-N,N-diethyl carbamate

* Examples from a larger series: *J. Nat. Cancer Inst.* 10: 1123, 1950.

This pattern, then, describes the dividing egg's response to carbamates and shows at least the visible mechanism involved. The pattern is typical of a number of carbamates. Table 1 shows a few possible configurations produced by substituting at one end or the other of the molecule. If, then, we get the same pattern of cytological response regardless of the alterations we make in the ends of the molecule, it appears logical, at least as a naïve first assumption, to say that the ends do not control the reaction that

affects cellular processes. One could resort to other means to justify the ends. Suppose, for instance, that the molecule hydrolyses and reacts at the carboxyl group, so the ester group is irrelevant. But the higher esters are more potent narcotics and division-inhibitors than the lower esters. Potency is increased by adding carbons at either end, at least up to ten carbons (1). This point needs no elaboration, inasmuch as the carbamates so neatly fit the pattern of non-specific hypnotics. Accordingly, this presentation is based on the supposition that the molecule works as an intact unit, and the argument will be developed that the simple carbamates act alike.

Temperature entered the picture as a result of comparison of responses

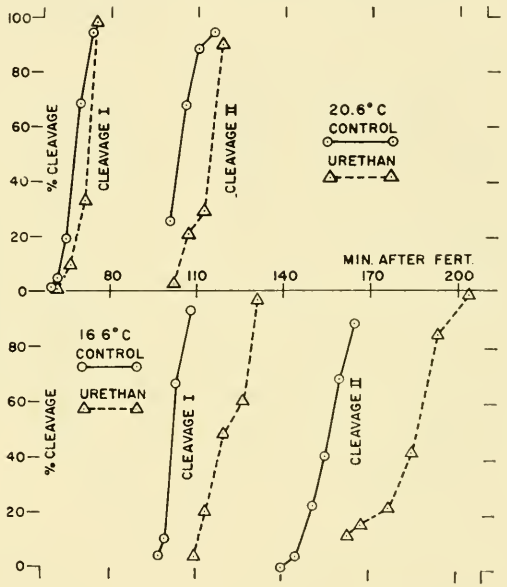


FIG. 2. *Arbacia* cleavage time in 55 mM urethan. First cleavage retarded 6% at 20.6° and 20% at 16.6°C.

of subtropical urchins such as *Tripneustes* and *Lytechinus* with those of *Arbacia* and the sand dollar, *Echinarachnius*. Temperature was a logical point of difference, so we set up water baths at an intermediate temperature that could be used at Woods Hole or Bermuda. These experiments were in part a screening project, and it would be convenient to refer all data to a common standard. Still the carbamates would not conform in different species, even at identical temperatures. The relationship between temperature and concentration of several carbamates in the two northern species, *Arbacia* and *Echinarachnius*, was subjected to more detailed analysis. Three concentrations were run simultaneously at three temperatures spaced at 4° intervals. The eggs were fertilized and at 10 minutes were placed in the carbamate solutions.

In figure 2 the effect of 4° differences in temperature is distinct in abso-

lute time and percentagewise: 6% and 12% for first and second cleavages at 20.6°C and 20% and 19% for the cleavages at 16.6°C. Further cooling made urethan even more effective, but extending the temperature above 20°–21° also increased the efficiency of urethan. From this it was concluded that around 20°–21°C, the cells were minimally susceptible to urethan.

Before generalizing, we must ask whether other carbamates imitate this urethan response to temperature as well as they do the cytological alterations. Comparison was limited to one carbamate with a completely substituted nitrogen: ethyl-N,N-diethyl carbamate. Figure 3 shows curves

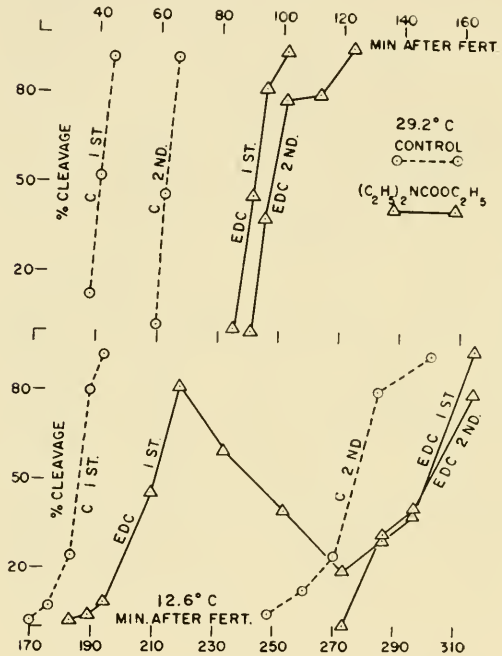


Fig. 3. Retardation and abortion of *Arbacia* cleavage by 6.6 mM diethyl urethan. At 12.6°C, 80% of the eggs formed deep temporary furrows that subsequently regressed. Successful 50% cleavage was delayed 64% beyond control cleavage at 12.6° and 127% beyond the controls at 29.2°C.

from two experiments. Simultaneously with the curves at the top, eggs were exposed at 25.2° and 21.2°. At 21° the retardation of first cleavage was 6½ minutes and second cleavage 11 minutes beyond the control 50% cleavage times of 60 minutes and 94 minutes. At 25° the retardation was 13 minutes for first cleavage and 18 minutes for second cleavage, compared to control 50% cleavages at 45 minutes and 69 minutes. Percentagewise, first cleavage delay was 11% at 21°, 29% at 25° and 127% at 29°. The curves at the bottom of the figure represent the coldest of a series that included 16.6° and 20.6°. In this series, delay by 0.1% diethyl urethan was about equal at 16.6° and 20.6°: 4 minutes at first cleavage and 10 to 12 at second. Percentage delay of first cleavage was 6% at 20.6°, 6% at 16.6°

and 14% at 12.6°. This calculation is based on the eggs' first try at division which was unsuccessful, producing the drop in cleavage percentage shown in the curve for 12.6°. These figures point to minimal sensitivity between 17° and 21°, or perhaps the curve flattens there. A series of finer temperature gradations are needed to pinpoint any flexure.

Converting the previous data to relate dose to a roughly equal retardation of the eggs produces figure 4. To induce a uniform retardation of division in sea-urchin eggs at temperatures from 12° to 21°–22°, one requires progressively more urethan—at least a twofold increase. Beyond 22°, the effectiveness of urethan again increases. Diethyl urethan fits this rough

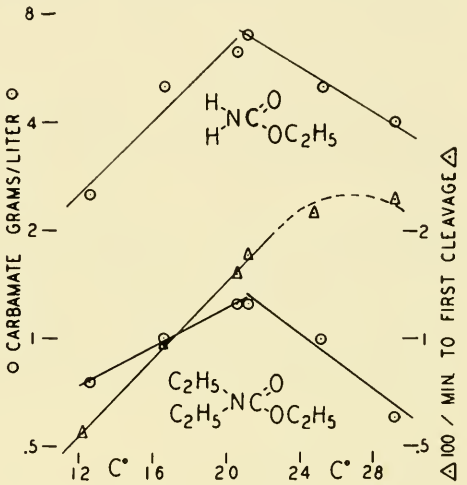


Fig. 4. Uniform retardation of *Arbacia* cleavage. Circles represent approximately equal retardation of cleavage at different temperatures and different doses (abscissa at left). To produce this effect, the maximum amount of ethyl carbamate (*upper curve*) or ethyl-N,N-diethyl carbamate (*lower curve*) is required at about 21°. Because the points are approximations, the slopes are not accurate. Cleavage rate (triangles, abscissa at right) reaches a maximum at 25°–26°C.

scheme. Although it is about six times as potent as urethan, it shows the same minimal effectiveness at 21°–22°.

These curves do not apply to the sand dollar. Compare, for instance, the marked retardation of *Echinarachnius* eggs at 22 mM and 20.2°C (fig. 1) as against slight retardation of *Arbacia* eggs by 55 mM at 20.6° (fig. 2). The difference lies not in the greater susceptibility of *Echinarachnius*, but in its different temperature response. It appears to be minimally affected by urethan somewhere below 17°C. Resistance to diethyl urethan also is shifted toward the lower temperature, so it appears that the curves for the two carbamates are roughly parallel, the diethyl congener being more effective throughout, as in *Arbacia*. The results for phenyl urethan (ethyl-N-phenyl-carbamate) do not fit this pattern. Possibly its low solubility introduces a complicating influence. There is, then, a species difference. It appears not to be random. *Echinarachnius* comes from colder water north of Cape Cod. It does not easily survive aquarium temperatures much above

20°, and egg cleavage also becomes abnormal above 20°. From this we generalized that the temperature at which urethan acts least on cell division lies close to the spawning temperature of the sea urchin. The data from *Tripneustes* and *Lytechinus* fit this supposition, but we do not have enough runs over a wide enough range to test it critically.

Coming back to the point of flexure in the dose-temperature curves, one can say only that it occurs at a temperature *Arbacia* prefers and the one at which it breeds. It is not the maximal rate of cleavage. If one plots rate of cleavage against temperature, the maximum falls somewhere around 26°C (fig. 4, right-hand ordinates). This cannot be called the optimal temperature if you include normal development in the criteria, for at 24°–28° cleavage is irregular in some eggs from the very beginning, and development is abnormal. Perhaps 21°–22° is optimal for *Arbacia* development, depending on what meaning you put on the word.

Because a wide variety of molecular configurations produces the typical carbamate response, and because urethan can be readily removed from the cells, it appears that the carbamates do not combine strongly with the protoplasmic constituents involved, but are loosely held in the cell by adsorption or solution (3). If the binding is of this latter type, then it follows that as the temperature is increased, the narcotic is less easily retained in the cell. Hence the direct relationship between concentration and temperature in the lower temperature range. Beyond the optimal temperature, however, heat itself produces untoward effects. Both aspects of the process can be related to a single mechanism if we assume the carbamate inactivates an enzyme, and that above a critical temperature, varying with the species, heat contributes to this enzyme inactivation (5). Cytological similarity of effects throughout the temperature range would support this single-process interpretation. The egg and cell division are too complex to permit delineation of the ultimate mechanism. It is hoped that these data will provide some transition, however, between the trenchant quantitations of enzyme systems and the bewildering biology of the intact organism.

SUMMARY

Carbamates, over a four-fold dose range, retarded the division of sea-urchin eggs without disrupting the ontogenetic process. *Echinarachnius* eggs at 20°C were slightly retarded by 11 mM urethan, and time to first cleavage was doubled at 22 mM. Carbamates of a great variety of alkyl and aryl substitutions also retarded cleavage in proportion to dose and, in general, the higher congeners were more effective than ethyl carbamate. In the two species most extensively studied, the effectiveness of carbamates varied with temperature. The first cleavage of *Arbacia* was retarded 6%

at 21°C, 17% at 17° and was partially suppressed at 13° in one series using 55 mM urethan. In another, 44 mM retarded 2% at 21°, 7% at 25° and 27% at 29°. Ethyl-N,N-diethyl carbamate also retarded minimally at 20°–21°. It was always more effective than urethan, 7 mM retarding first cleavage 10% at 21°. *Echinarachnius* also showed these two separate trends in response to temperature, but the break in the curve came at 16°. It is proposed that the carbamate series are typical nonspecific hypnotics, held loosely in the cell by some physical mechanism. The carbamate molecules are less readily captured by the cell components at higher temperatures, but beyond a critical temperature, heat accelerates the process promoted by the carbamates. Denaturation of a single enzyme would fit this picture, but the system is too complex to yield a definitive answer on the basis of the evidence at hand.

REFERENCES

1. Cornman, I. Inhibition of sea-urchin egg cleavage by a series of substituted carbamates. *J. Nat. Cancer Inst.* 10: 1123–1138, 1950.
2. Cornman, I. The properties of urethan considered in relation to its action on mitosis. *Internat. Rev. Cytol.* 3: 113–130, 1954.
3. Cornman, I., H. E. Skipper and J. H. Mitchell. The fixation of urethan carbon atoms in sperm and in resting and rapidly dividing cells. *Cancer Res.* 11: 195–199, 1951.
4. Harvey, Ethel B. *The American Arbacia and Other Sea Urchins.* Princeton, N. J.: Princeton, 1956.
5. Johnson, F. H., H. Eyring and M. J. Polissar. *The Kinetic Basis of Molecular Biology.* New York: Wiley, 1954.

CUMULATIVE EFFECTS OF OPTIMUM AND SUBOPTIMUM TEMPERATURES ON INSECT DEVELOPMENT[1]

A. Glenn Richards, *Department of Entomology and Economic Zoology, University of Minnesota, St. Paul, Minnesota*

The voluminous literature on the effects of temperature on development in general or on insect development in particular leaves numerous questions unanswered (1–3, 9). The present paper will deal with two of these questions, namely, why is the hatching threshold several degrees above the temperature at which the developmental rate approaches zero; and why are insects which hatch under near minimal conditions so debilitated that they subsequently die even though placed under optimal conditions?

As experimental material for this study a laboratory colony of the Large Milkweed Bug, *Oncopeltus fasciatus* (Dallas) (Order Hemiptera), was used. This species gives the type of growth curves commonly obtained from insects (8). The time-temperature curve for hatching (fig. 1, curve A) is nearly a hyperbola but discrepancies from the hyperbolic form are well shown by the reciprocal, the rate-temperature curve (fig. 1, curve B), which is clearly not a straight line. Various attempts have been made to find an algebraic expression satisfying these curves. Thus, Browning (4) finds closer fit with a logistic curve and Huffaker (6), with a catenary curve. It does not seem worthwhile to discuss the application of these various equations since there is no sound basis for thinking any one of them gives a portrayal of relevant phenomena. Their appearance in the literature seems to be no more than the outcome of an empirical search for a mathematical model that will produce a curve of this shape, and any correspondence found might be fortuitous.

However, it should be said that the search for a satisfactory equation has a practical basis which makes fortuitousness of fit immaterial. It would be exteremely useful, especially for ecological work in the field, to have an equation by means of which one could calculate the hatching threshold from a few points without the laborious laboratory work involved in empirical determination of this threshold. Unfortunately, like previous investigators, we have not found it possible to approximate this

[1] Paper No. 3615, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul 1, Minn.

temperature threshold by application of any algebraic formula to time-temperature curves.

Another question frequently treated in past literature is whether or not the summation aspect of temperature is the same for constant and varying temperature conditions. This is the familiar 'day-degrees accumulation,' widely used to forecast probable dates of hatching, of pest outbreaks, of flowering and harvesting, and even of the amount of fuel needed to heat a building during winters of different degrees of coldness. Genesis of this idea dates back to Reaumur in the first half of the 18th century. A large body of data attests the adequacy of these arithmetric calculations when the different temperatures are above developmental threshold. As an ex-

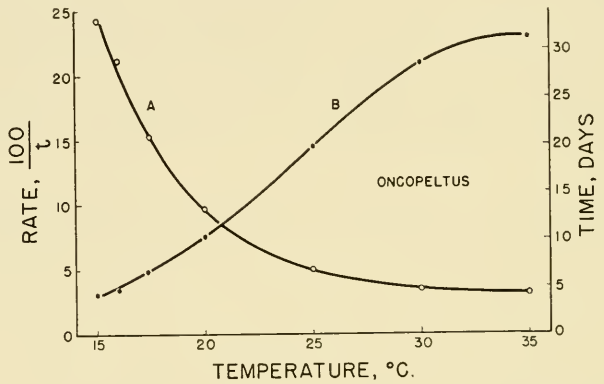


Fig. 1. Development of *Oncopeltus fasciatus* (Dallas) eggs through hatching for the viable range of temperatures. A, time-temperature curve; B, rate-temperature curve. After Lin *et al.*, 8.

ample, this is well shown by *Oncopeltus* eggs when they are incubated for various periods at 15°C and then transferred to 30°C (fig. 2, curve B). There is an essentially linear decrease in time required at the higher temperature when the eggs are held for longer periods at the lower temperature. Little literature deals with summation effects when one of the temperatures is below the constant temperature threshold, but here again we find linear decreases indicating that summation effects can be calculated provided the subthreshold temperature is only a little below the threshold (fig. 3, curve B).

The whole concept of temperature thresholds for development, at least in insects, has lacked definiteness, perhaps because of the complexity of phenomena involved (8). Perhaps the most useful definition is "... the lowest temperature at which a given physiological process, or development through a given stage in the life history, can be carried through to com-

pletion" (1). This definition implies that there is a series of thresholds in development of any species. Experimentally this is indeed found to be

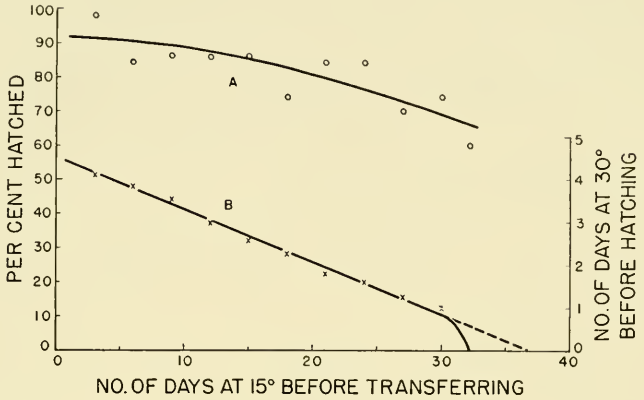


Fig. 2. Development of *Oncopeltus* eggs at 30° following various lengths of incubation at 15°C. A, percentage hatching at 30°; B, time required to hatch after transferal to 30°. After Lin *et al.*, 8.

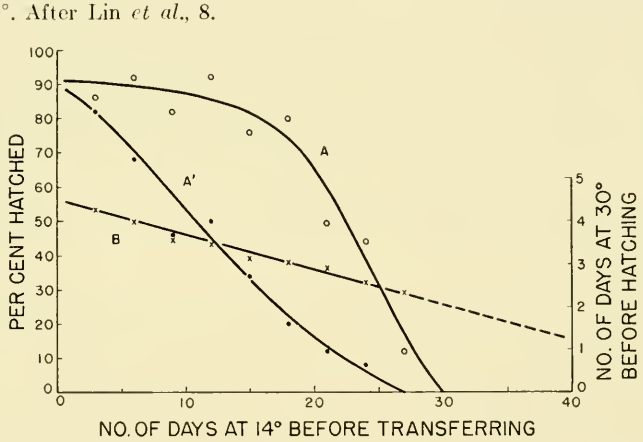


Fig. 3. Development of *Oncopeltus* eggs at 30° following various lengths of incubation at the subthreshold temperature of 14°C. Hatching does not occur at any temperature if the exposure to 14° is for more than 27–28 days. A, A', per cent hatching at 30° in two separate tests; B, time required to hatch after transferal to 30°. After Lin *et al.*, 8.

correct. Johnson (7) defined three different thresholds for the egg stage of the bedbug: a 'developmental threshold' below which no embryonic differentiation occurred, a 'hatching threshold' below which no hatching occurred, and a 'developmental-hatching threshold' below which development would not be followed by hatching. He found these three thresholds

to be at 4°, 8° and 13°C, respectively. Similar thresholds can be defined for *Oncopeltus* where development to blastoderm occurs at 5°C, development to a complete nymph requires a little above 10°C, hatching requires 13°C, complete development plus hatching requires 15°C, and full viability of the hatched bug requires something above 17°C (5). A longer series of thresholds could be defined if one wished to do the necessary work. The first threshold which will be dealt with here is the 'developmental-hatching threshold' of Johnson but for simplicity of expression it will henceforth be called the 'hatching threshold.'

One of the most serious limitations to series of thresholds, such as those above, is that they are determined on a basis of incubation at constant temperatures or (for the 'hatching threshold' of Johnson) incubation at one constant temperature followed by a single shift to another constant temperature. Determinations of threshold values are repeatable only under identical conditions. Thus we find that if one uses varying temperatures instead of constant temperatures (such as several hours at 20° or 25° followed by the remainder of each day at 13° or 14°) the thresholds when stated as average temperatures are found to be 1–2°C lower (8). Another limitation we find is that the hatching temperature threshold is not independent of all other factors. If the relative humidity is lowered from the optimal value of about 75% to 50%, hatching is poorer or, otherwise stated, the temperature for the hatching threshold is raised several degrees (8). One may object to comparing the effects of an average temperature with those of its corresponding constant temperature, or to modifying other environmental conditions, but clearly the thresholds are difficult to define satisfactorily and can be defined only in terms of a fully stated set of conditions.

Incidentally, no one of these thresholds in *Oncopeltus* corresponds to the chill-coma temperature, which for just-hatched bugs is 3°, or for bugs a day or two after hatching is 2°C.

Confining our attention for the moment to the hatching threshold (*i.e.* that temperature which when maintained constantly with optimal humidity will just permit full embryonic development plus hatching) we start by acknowledging the correctness of the day-degree accumulation idea. The developing embryo acts like an adding machine. It summates rate at particular temperatures × time at those temperatures, and hatches when the calculated 100% of developmental time has been attained (8). Discrepancies occur in alternating temperature experiments, especially when one of the temperatures is subthreshold. A large part of the larger errors, however, is readily ascribable to uncertainty about the determination of rate values to use for subthreshold temperatures (shown by the >10%

discrepancy between expected and obtained value for development at 15°; see fig. 2, curve B).

But if the day-degree accumulation idea is correct, why is there a finite threshold well removed from zero developmental rate? This threshold is sharp for constant temperature experiments: about one-third the normal hatching percentage occurs at 15°, but zero percentage at 14°C. The threshold is also sharp for alternating temperature experiments when only two or three temperature shifts are made. In early stages of development, *Oncopeltus* eggs will tolerate 35%–45% of the developmental time at below threshold; in later stages they will tolerate 25%–35% of developmental time at below threshold. The effect is sharp, practically a cut-off (fig. 4), and nearly independent of when the subthreshold temperature treatment is

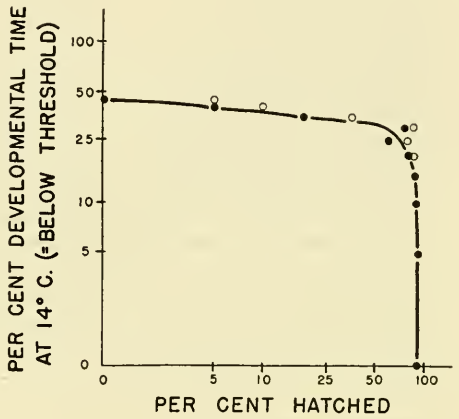
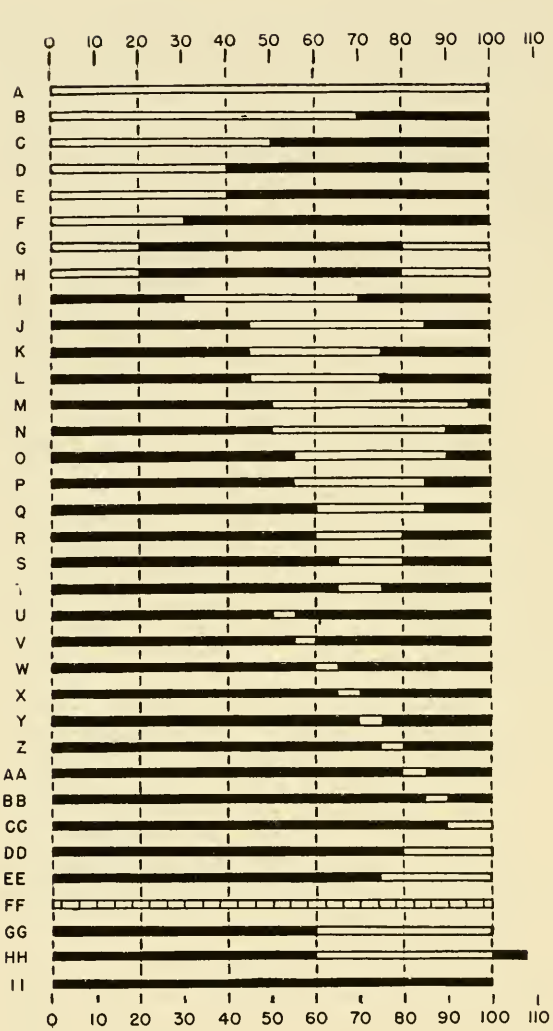


Fig. 4. Plot showing relation between hatching and length of time at a subthreshold temperature for *Oncopeltus* eggs. *Solid circles:* percentages fully hatched; *open circles:* same plus those that ruptured the egg shell without emerging completely ('semi-hatched'). After Lin *et al.*, 8.

given (fig. 5). Clearly, there is no 'sensitive period', i.e. no one stage in embryonic development which has a threshold requiring for its completion a temperature higher than that of the hatching threshold.

A number of other possibilities have been eliminated (8). Thus, the periodicity of frequent temperature alternations is not important as shown by alternations arranged on the basis of a table of random numbers. Time itself is not important since it can be varied widely by the use of various combinations of three or four different temperatures which result in the same developmental time being required although the actual time varies considerably. Another obvious suggestion is that the hatching process itself is critical but we find that if most of development has been undergone at 20° or 25°, hatching will readily occur at temperatures several degrees below the normal or constant-temperature hatching threshold (this agrees with Johnson's (7) data for the bedbug). In fact, numerous attempts to



	% HATCH	% DIED HATCHING	% DIED PRIOR TO HATCHING
A	0	0	100
B	0	FEW	MOST
C	0	FEW	MOST
D	40 ± 4	13.5	46.5
E	15 ± 3	15	70
F	88	—	—
G	9 ± 2.6	6.5	85
H	2.5 ± 1.3	6	91
I	43 ± 5	40	17
J	7.5 ± 2	38	55
K	44 ± 7	28	28
L	40 ± 6	—	—
M	0	5	95
N	5 ± 2	5	90
O	18 ± 8	18	64
P	75 ± 4	11	14
Q	60 ± 6	17	23
R	79 ± 4	6	15
S	89 ± 3	3	8
T	89 ± 3	5	6
U	94 ± 1	0	6
V	90 ± 3	0	10
W	88 ± 3	0	12
X	96 ± 2	0	4
Y	92 ± 4	1	7
Z	95 ± 3	1	4
AA	94 ± 3	0	6
BB	96 ± 2	0	4
CC	80	—	—
DD	31	—	—
EE	0	FEW	MOST
FF	88	4	8
GG	0	0	100
HH	2 ± 1.3	7	91
II	90–98	0	4–10

FIG. 5. Diagrammatic presentation of data demonstrating absence of a 'sensitive period' for the deleterious effect of low temperature on *Oncopeltus* eggs. The time scale is 'developmental time,' *i.e.* actual time × developmental rate at that temperature. Each line is a separate experiment. *Open bars:* per cent developmental time at the subthreshold temperature of 14°; *solid bars:* per cent developmental time at the favorable temperature of 20° or 25°. Modified after Lin *et al.*, 8.

find anything critical or limiting in the hatching process of *Oncopeltus* failed; debilitated young bugs died before, during or after hatching with no discernible relationship to the hatching process itself.

Since no specific explanation of results was located in tests such as those itemized in the above paragraph, we returned to an analysis of the time-temperature curves. No useful ideas came from curves such as those shown in figures 1–3. Expressions concerning them seem no more than juggling algebraic or arithmetric terms—even though they have practical use for forecasting they give no clue as to what is added or how it is added. The situation can hardly be as simple as the mere summation of similarly activated chemical events because this would leave unexplained the >10% discrepancy at the end of curve B in figure 2. A clue is forthcoming if we

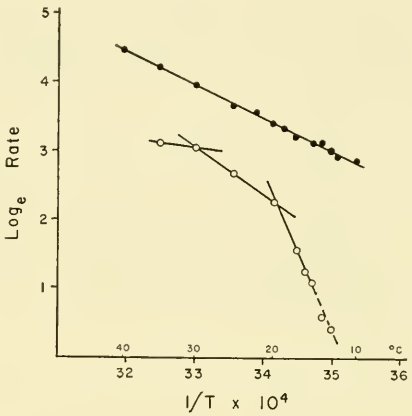


FIG. 6. Comparison of oxygen consumption of recently hatched *Oncopeltus* larvae (*solid circles*) with the developmental rate of *Oncopeltus* eggs (*open circles*). The developmental rate curve is drawn as a broken line for the calculated values at subthreshold temperatures. Actual rates of development available in fig. 1; O_2 consumption at 30°C is 0.39 mm^3/larva/hr.

re-plot the developmental rate data as an Arrhenius-type plot and compare it with a similar plot for oxygen consumption (fig. 6). (No attempt will be made to justify the drawing of a series of straight lines through the points for developmental rate—it is so drawn here for illustrative purposes.) Whether one draws straight lines or smooth curves through these points, the fact remains that oxygen consumption, which is linear throughout more than the viable range, nearly parallels developmental rate in the range 20°–30° but deviates widely from it below 20°C.[2] It follows from these curves that progressively more and more energy should be required to complete development as the temperature is lowered further below 20°C.

The above is substantiated by measurement of weight losses following incubation at 25°, 17° and 15°C (fig. 7 and table 1). Ignoring the variation

[2] If one calculates from the straight lines drawn, the developmental rate has a μ value of about 13,600 from 20° to 30°C, and about 46,000 below 20°C.

TABLE 1. WEIGHTS OF FRESH EGGS AND OF LARVAE OF ONCOPELTUS INCUBATED AT THE INDICATED TEMPERATURES*

	NO. OF SAMPLES	AV. LIVE WT.	AV. DRY WT.	AV. ETHER EXTRACT	AV. RESIDUE
1. Fresh eggs	6	236.4 ± 3.4	74.6 ± 0.66	27.7 ± 0.61	46.8 ± 0.93
25° incubation	6	210.6 ± 4.5	63.7 ± 1.3	18.0 ± 0.47	46.0 ± 1.1
17° incubation	6	194.5 ± 1.6	58.0 ± 0.57	14.8 ± 0.53	42.8 ± 0.68
2. Fresh eggs	20	255.4 ± 3.6			
25° incubation	20	224.8 ± 3.2			
17° incubation	20	203.2 ± 2.7			
3. Fresh eggs	1	273	(82)†	(30.5)†	(52)†
15° incubation	1	190	59.6	13	48

* Average weights in μg/individual from lots of usually 200–600, with standard error calculated from variation between samples. Lot no. 1 from fig. 7 A–C; lot no. 2 from fig. 7 D; lot no. 3 a single batch of 580 eggs.

† Approximate values calculated from percentages determined for lot no. 1.

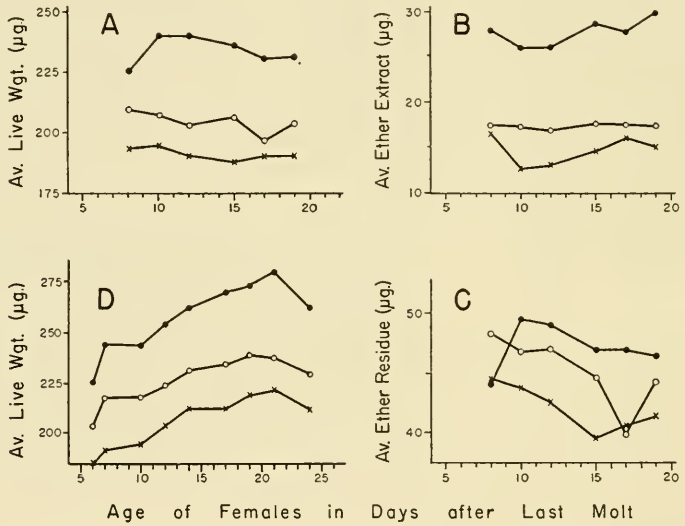


FIG. 7. Comparison of weights of freshly laid *Oncopeltus* eggs (*solid circles*) with the weights of larvae incubated at 25° (*open circles*) and 17° (*crosses*). A, live weights of six lots of eggs and larvae from one lot of parents; B, weights of ether extracts from these; C, weights of dry residues from same; D, repeat experiment with randomization better controlled by incubating the eggs weighed instead of using separate egg lots for each curve (because of space, data from only first 9 of 20 egg lots plotted here).

in egg weight as a function of the female's age and treating the separate points as simply so many replicates, one would expect these curves to be parallel. The fact that they are only roughly parallel is at least largely due to individual variation and inadequate randomization of the some-

what sticky eggs. This reasonable interpretation is supported by the better paralleling of curves in figure 7D where the weighed egg lots were divided into two parts for incubation at the two temperatures, whereas the extractions being performed necessitated the use of separate egg lots for the curves in figures 7A–C. (See also 8A.)

It is well known that the oxygen consumption of insect eggs increases gradually through development and then rises sharply at the time of hatching. It seemed unnecessarily laborious to determine total oxygen consumption throughout development at various temperatures. The values given in figure 6 are based on recently hatched larvae. Making the assumption that the shape of the curve for oxygen consumption versus developmental stage will be similar for all temperatures, the values from the recently hatched larvae can be used to obtain ratios of expected energy utilization at various temperatures.[3] These ratios are calculated in table 2 both on the basis of a μ value of 10,000 for oxygen consumption (this is the line drawn in fig. 6) and on the basis of a μ value of 14,000 (the value obtained in an earlier independent determination plotted in fig. 10). Since the water content remains constant (measured values 68%–71%) one would expect agreement between ratios of dry weight losses and oxygen consumption. Agreement is closely approximated at constant incubation temperatures if the oxygen consumption has a μ value of 14,000; less closely but still approximately if the μ value is 10,000. The data suggest that when incubation is at constant temperatures, the day-degree accumulation will be equivalent to calorie-counting.

Since we have at present no way of estimating how much loss of dry weight can be tolerated, more interest centers on fractionation of the dry weight loss. The average amounts of ether extract are 26–30 μg/egg for eggs with live weight averages of 225–240 μg. Somewhat less than half of this ether extract is lost in incubation at 25°, about half at 17°, about two-thirds at 15° and by extrapolation nearly complete loss would be expected at 14°. But a Soxhlet extraction does not distinguish between fats which are present as available food reserves, and fats of structural importance such as cuticular waxes, nerve sheaths, etc. To a first approximation it seems reasonable to conclude that on the average the fat reserve available for energy is nearly exhausted following incubation at 15° and would be inadequate for incubation at 14°. At the lower temperatures there are also significant losses in the ether residue fraction but we have at present no data to show whether this is due to loss of carbohydrate or protein or something else.

Further support for the preceding interpretation comes from a consider-

[3] The energy expenditure to produce a larva can be calculated approximately from the measured weight losses. It is only a few tenths of a gram calorie at 25°C.

ation of larvae which had been incubated at the hatching threshold temperature of 15°C. Weight data from one lot of 580 eggs which gave the low hatch of only 8% could be stated as showing that bugs which only semi-hatched used up more than 1.5× as much material as those that gave active larvae (table 3). This seems unreasonable, especially since we have data showing that individual egg weights vary considerably within a single lot of eggs, and since larvae lose approximately the same amount of weight

TABLE 2. DETERMINED AND CALCULATED WEIGHT LOSSES OF LARVAE OF ONCOPELTUS INCUBATED AT VARIOUS TEMPERATURES, COMPARED WITH LOSS RATIOS CALCULATED FROM OXYGEN CONSUMPTION

INCUBATION TEMP. °C	$\frac{O_2 \text{ AT } T_X \times \text{TIME}}{O_2 \text{ AT } 25° \times \text{TIME}}$		LOSS LIVE WT.		LOSS DRY WT.		LOSS ETHER EXTRACT		LOSS RESIDUE	
	With μ for O_2 at 10000	With μ for O_2 at 14000	Measured	Ratio	Measured	Ratio	Measured	Ratio	Measured	Ratio
35	1.08	1.48								
30	0.91	1.11								
25	1.00	1.00	a = 25.8	1.00	10.9	1.00	9.7	1.00	0.8	1.00
			b = 30.6	1.00	(9.5)	1.00				
20	1.43	1.35								
17	1.91	1.68	a = 41.9	1.6 ×	16.6	1.5 ×	12.9	1.35 ×	4.0	5 ×
			b = 52.2	1.7 ×	(16)	(1.7 ×)				
16	2.52	2.12								
15	2.75	2.24	c = 83	2.5 ×*	22.4	2.1 ×*	*ca* 18	1.7 ×*	4–5	5–6 ×
14	3.56	2.83	(109)†		(27)					
13	4.46	3.44	(136)†		(32)					
av. 17‡	1.91	1.68	43	1.35 ×						
av. 15‡	2.75	2.24	45	1.4 ×						

Data from table 1 and fig. 6; weights in average μg/larva. Calculated values in parentheses.

* Corrected by factor of 255/273; see weights in table 1.

† Calculated on basis of 'b' set using ratios 3.56 and 4.46.

‡ Average temperatures obtained by 2 hr. at 25° + 22 hr. at 15° daily, and 2 hr. at 25° + 22 hr. at 13° daily, respectively.

after incubation at either 17° or 25° irrespective of the original egg weight (fig. 7A, D). If we assume that all the embryos being incubated at 15° use about the same amount of energy, then those that successfully hatched would have average dry weights of 67.5 + 22.4 = 89.8 μg which corresponds to a live weight of about 280 μg, whereas those that did not quite succeed in hatching would have average dryweights of 58.0 + 22.4 = 80.4 μg which corresponds to a live weight of a little less than 260 μg. This amount of variation is not excessive for individual egg weights within a lot of 580 eggs. The obvious conclusion, then, is that at the threshold tem-

perature only the heavier eggs have enough available stored reserves to go through hatching.

The above correlation is so reminiscent of variation found by Lin *et al.* (8) in hatching percentage curves (fig. 3, curves A and A′) that the present author is inclined to go a step further and to suggest that the wide variation in hatching curves at minimal conditions is primarily due to variation in egg size in different samples of eggs.

If the hatching threshold is simply an expression of the point at which exhaustion of available reserves occurs one would not necessarily expect to find any organ or tissue pathology associated with the threshold—and inspection of serial sections stained in various ways revealed no pathology.

TABLE 3. MEASURED AND ESTIMATED VALUES FOR VARIATION IN RESULTS OF INCUBATION OF ONCOPELTUS AT THRESHOLD TEMPERATURE OF 15° C*

MATERIAL	AV. LIVE WT.	AV. DRY WT.	AV. ETHER EXTRACT	AV. RESIDUE
580 fresh eggs	273	(82)	(30)	(52)
40 active larvae	212	67.5		58
200 semi-hatched	186	58.0	12	46
Weighted averages	190	59.6		48
	LOSS LIVE WT.	LOSS DRY WT.	LOSS ETHER EXTRACT	RESIDUE CHANGE
40 active larvae	61	14.5	(10)	+6
200 semi-hatched	87	24	18	−6
Weighted average	83	22.4	17–18	−4–5

* Weights in micrograms. Estimated values given in parentheses, see table 1.

Implicit in the above presentation is the idea that the stored reserves are completely exhausted at subthreshold temperatures. Direct histological examination shows this is *not* true. Serial sections show that larvae unsuccessful at hatching at 15° (some of the semi-hatched ones of table 3) still had considerable quantities of yolk enclosed in the midgut (fig. 8). And extirpated midguts showed a little Sudan staining. Calculations show that the fat loss at 14° should equal or exceed the amount available but there would presumably still be some albuminous yolk present. Since this histological evidence shows that the stored reserves are not completely exhausted, and since eggs can hatch at average temperatures of 14° although not at a constant temperature of 14° (see table 4), further work will be necessary to determine whether some particular component of the food reserves is exhausted or whether the phenomenon is complicated by some additional factors such as that to be discussed in the next section.

In conclusion on this portion of the paper, we have eliminated numerous possibilities as to why the hatching threshold is several degrees above the temperature at which the developmental rate approaches zero. To a first approximation there is a good correlation between this threshold and the depletion of stored food reserves due to the different effect of temperature on development versus energy expenditure below 20°C. But recent data suggest that this explanation is too simple and will require further elaboration.

Turning to the second question posed at the beginning of this paper, we are faced with the puzzling situation that with the change in developmental rate below 20°C (fig. 6) we can still get hatching, even very good hatching,

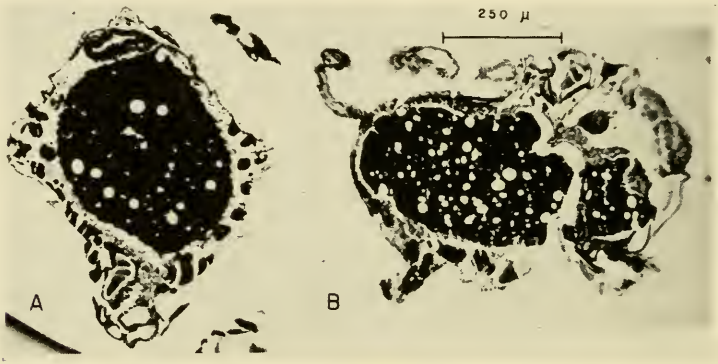


FIG. 8. Low magnification photomicrographs of *Oncopeltus* larvae incompletely hatched after incubation at 15°C. Show presence of large mass of albuminous yolk in the midgut (these semi-hatched larvae had died and partially dried prior to placement in the fixing fluid; hence tissue details are poor). A, cross section through midgut region; B, longitudinal frontal section. Iron hematoxylin stain.

but the larvae are in some way debilitated so that none, or only a much lower percentage than normal, grow to maturity even when they are immediately transferred to optimum conditions. It seems that we have here a case where there is some biological significance to what Crozier would have called a 'critical temperature' in the developmental rate curve. This phenomenon of a 'viability threshold' was discovered in connection with an unsuccessful attempt to isolate a strain of *Oncopeltus* with lower temperature thresholds (8); it has been considerably extended by Hodson and Al Rawy (5). Their extensive data are summarized in figure 9. In examining figure 9 one must remember that the plotted percentages show survival under favorable conditions of larvae from eggs incubated at the indicated conditions of temperature and humidity. Another way of saying this is that the temperature and humidity values given beneath the bars represent

pretreatment conditions, percentages on the vertical axis represent hatching under these conditions and subsequent survival under a favorable temperature. Note that, for incubation at the above threshold temperature of 17°, they obtained an excellent hatch (85%) but even though the larvae were immediately placed at 25° the bugs all died. In the author's laboratory variable but relatively low survival values have been obtained (table 4). Whether this difference between their results and ours is related to dif-

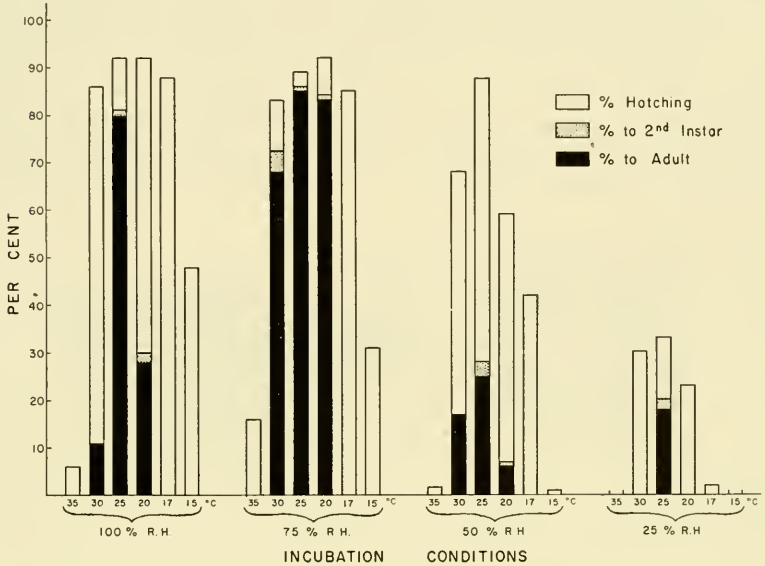


Fig. 9. Bar diagram giving hatching percentages of *Oncopeltus* eggs under stated incubation conditions, and percentages of those hatching that subsequently matured under standard favorable conditions of 25°C and 50% R. H. After Hodson and Al Rawy, 5.

ferent humidities or to some other factor remains to be determined but both sets of data show much lower than normal survival.

Our attempts to determine the nature of this debility induced by a low temperature that does not necessarily impede hatching have been largely negative to date. Thus, there is no significant difference between the oxygen consumption of recently hatched larvae from eggs incubated at 17° and 25° (fig. 10). On a per larva basis the 25° individuals give slightly higher oxygen consumptions; on a weight basis they give slightly lower ones. There may still be a difference in oxidative metabolism because preliminary determinations of the succinoxidase activity of brei (with exogenous succinate and cytochrome *c*) suggests a 2× greater activity from eggs incubated at 25°C. Further study of this is needed.

There is no noticeable difference in the color or melting points of fats from 17° and 25° larvae. The ether extract is a brownish amber and begins softening at about 30° but does not fully melt until 50–55°C.[4] The debility is certainly not caused by exhaustion of total food reserves because larvae from eggs incubated at 17° still have about half the original fat of the egg and a large amount of the albuminous yolk. Even after incubation at 15° the larvae contain some fat and considerable albuminous yolk (fig. 8). These gross determinations, however, do not exclude the possibility that the important factor may be exhaustion of some specific component in the stored reserves. To test this, several lots of larvae from eggs incubated at 17° were induced to feed on solutions containing glucose, a mixture of known vitamins, yeast or casein, or a mixture of all of these (in addition to the availability of milkweed seeds). The low percentage survival was no better on these fortified diets than in the controls reared under standard culture

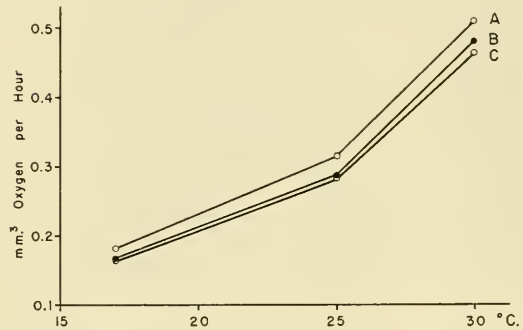


Fig. 10. Oxygen consumption of freshly hatched *Oncopeltus* larvae incubated at 17° (*open circles*) and 25° (*solid circles*). A, values of curve C corrected to a comparable weight basis by multiplying by 1.1; B and C, average O_2 consumption per larva.

conditions. Since we were not successful in inducing vitality by this means, I doubt that the debility has a nutritional deficiency basis.

The conclusion that the debility does not have a nutritional basis is supported by the finding that larvae from eggs incubated at 25° gradually die off when reared at 17°C. From 257 larvae, a single male adult was obtained after 103 days; yet these larvae fed on the normal culture food. Incidentally, the single adult obtained was one which had spent its entire growth period (125 days) at 17°; none of the 177 larvae from eggs incubated at 25° survived past the last larval instar.

The debility also does not have the aspect of having a hormonal basis. To be such, the hormone would have to be a general growth promoter present from fairly early embryonic stages (not later than the time when

[4] This contrasts with the ether extract from freshly laid eggs. Egg fats are a yellow amber and fluid at room temperature. But we do not know whether this difference indicates a metabolic change or only a changing proportion due to the production of cuticular waxes.

organ segregation is being completed) through into the adult stage. No such omnipresent hormone is known.

Finally, we found no visible pathology even at the mitochondrial level. Tissues of all organs were examined in serial sections after staining with Heidenhain's iron hematoxylin, the Feulgen reaction, the periodic acid-Schiff reaction for polysaccharides and aniline acid fuchsin-methyl green for mitochondria. Whole mounts were also examined after staining with Black Sudan B. Because of the interest to oxidative metabolism, these negative results are illustrated by photographs of mitochondria in oenocytes of larvae from eggs incubated at 17° and 25° (fig. 11). Clearly, the mitochondria appear equally normal in both.

But we do have one clue. The debility can be overcome in either embryonic or larval stages by warming the animals for a few hours daily (fig. 5, line FF, and table 4). In other words, if alternation from favorable (20°–

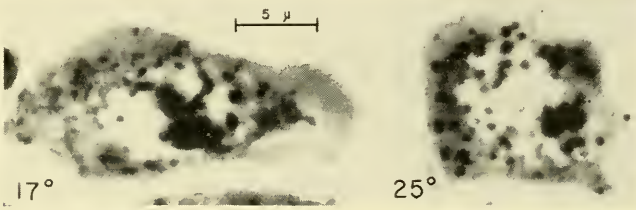


Fig. 11. Photomicrographs of sections of freshly hatched *Oncopeltus* larvae incubated at 17° and 25° C showing similarity of the mitochondria in oenocytes. (Zirkle's fixative, aniline acid fuchsin-methyl green stain, photographed with green filter.)

30°C) to subthreshold temperature (13°–14°C) is made only once or a few times, then some 60%–70% of developmental time (= >20% of actual time) must be at the favorable temperature to produce hatching (more to produce viable larvae), but if the warm treatment is given in frequently repeated small doses then less than 40% of developmental time (= <10% of actual time) will produce both fair hatching and fair viability in those that hatch. At favorable temperatures, that amount of time which when given as short daily treatments will produce normal hatching and normal viability (survival), will when given as a single warm treatment produce a small hatching percentage with zero or nearly zero viability. Also note that an average temperature of 15° gives much more viability when the average temperature is produced by 4 hours at 25° plus 20 hours at 13° than when it is produced by 2 hours at 25° plus 22 hours at 14°. It follows that the average temperature is not of primary importance. When warm treatments are given at frequent intervals, the length of time at the favorable temperature seems more important than the over-all temperature average.

There is another unexpected feature to the alternating temperature experiments, namely that the weight losses at average incubation temperatures of 15° and 17° are not as great as those at constant incubation temperatures of 15° and 17° (table 2). Correlated with this lower weight loss there is good hatching (86% and 88%) and high vitality (60% and 78% survival). One way to summarize the differences in effects of constant

TABLE 4. PERCENTAGE SUCCESSFUL GROWTH OF ONCOPELTUS LARVAE FOLLOWING INCUBATION UNDER VARIOUS CONDITIONS

INCUBATION CONDITIONS		% HATCH + SEMI-HATCH	% THAT GROW AFTER HATCHING			
Temp. °C	R.H.		Temp.	R.H.	Molt once	Grow to adult
25	75	80–95	25	75		70–95
25	75	90	25	50[1]	76	75
25	50[1]	88	25	50[1]	28	25
25	75	90	17	75	16	0.4
17	75	76	25	75		0–30
17	75	85	25	50[1]	0	0
av. 17[2]	75	86	25	75		60
av. 16.4[3]	75	14 + 23	25	75	0.5	0.2
15	75	25 + 30	25	75	0	0
av. 15[4]	75	63 + 26	25	75	49	37
av. 15[5]	75	88	25	75		78
14	75	0				
av. 14[6]	75	78				
av. 14[7]	75	62				

[1] Data from 50% R. H. taken from Hodson and Al Rawy (5).

[2] Av. 17° produced by 4 hr. at 25° + 20 hr. at 15° daily.

[3] Av. 16.4° produced by 2¼ days at 25°, then 19¼ days at 14°, then 2¼ days at 25°. Similar to line I of fig. 5. Included to show necessity of frequent warming. (From ref. 8.)

[4] Av. 15° produced by 2 hr. at 25° + 22 hr. at 14° daily.

[5] Av. 15° produced by 4 hr. at 25° + 20 hr. at 13° daily.

[6] Av. 14° produced by 4 hr. at 20° + 20 hr. at 13° daily. (From ref. 8.)

[7] Av. 14° produced by 2 hr. at 25° + 22 hr. at 13° daily. (From ref. 8.)

versus alternating temperatures is to say that the 'day-degree' calculation works satisfactorily for predicting the time of hatching under either constant or varying temperature conditions, but that the 'calorie-counter' calculation works satisfactorily for predicting weight losses only under constant incubation temperatures, and that neither at the present stage of investigation permits predicting viability.[5]

[5] It is of considerable ecological interest that developing *Oncopeltus* fare much better under varying temperature conditions than under a constant temperature which is equal to the average temperature of the varying set. It would seem both premature and out of place, however, to expand on this subject here.

Obviously there is something fundamentally different about development of *Oncopeltus* embryos and larvae at temperatures above and below 19°–20°C. But we have no idea what this difference is. Since for viability only a small portion of either actual or developmental time is needed at temperatures above 20°—provided such temperatures are supplied frequently and repeatedly throughout development—we are postulating as a working hypothesis that some vital factor (a 'vitality factor' if you wish to call it such) is not synthesized in adequate amounts at temperatures below 20°C. This would make the debility due to a biochemical deficiency in the metabolic machinery. However, it has been pointed out by others that the available data do not eliminate alternative possibilities such as the production or inadequate destruction of an inhibitor, or even physicochemical changes such as sol-gel transformations (compare article by Marsland in this volume).

A final warning should be made in connection with any serious discussion of the possibilities listed in the above paragraph. The present paper has been concerned exclusively with temperature effects throughout, and hence it may have given the impression that these effects are known to be due specifically to temperature. However, data in figure 9 show that mortality can be produced by other suboptimum conditions, such as humidity. It would be completely gratuitous to assume that the physiological effects of low temperature and low humidity are the same when the data are only mortality percentages. But it would also be unwise to assume that the debilitation is specifically and solely an effect of suboptimal temperatures—the debility could conceivably be a result of some general stress that in turn might be produced in various ways.

SUMMARY

Extending our previous work, we find that to a first approximation the occurrence of a finite temperature threshold for hatching of *Oncopeltus* eggs is correlated with depletion of food reserves brought about by the fact that temperatures below 20°C retard development much more than they retard expenditure of energy (fig. 6). Experiments with varying temperatures suggest that the above explanation is too simple and will require further elaboration. Variation in hatching percentage determinations at minimal temperatures is correlated with and presumably due to variation in individual egg sizes and hence to variation in amounts of food reserves in different eggs. In addition to the 'hatching threshold' there is what may be called a 'vitality threshold' which is independent of hatching and which requires temperatures of 20°C or higher. The requirements of this threshold can be satisfied with a small percentage of developmental time at temperatures above the threshold, provided the warmth is supplied as frequent short treatments. Possible bases for this effect are discussed.

The 'vitality threshold' seems to be correlated with the abrupt change in developmental rate at about 20°C. Calculating the time requirement for development to hatching works well by the classical day-degree accumulation method; calculating the energy or food requirement by the calorie-counter method works well only under constant temperature conditions of incubation, but neither calculation works for predicting vitality.

Acknowledgement is due for financial assistance received in support of this work from the National Science Foundation. The author's sincere personal thanks are due to his assistant, Marjorie Q. Kolderie for help throughout all stages of this study, and to graduate student assistants John De Witt and James Baird for making some determinations. My utmost appreciation is due to my colleague, Dr. A. C. Hodson, for his cooperation and for many discussions about this research.

REFERENCES

1. Allee, W. C., A. E. Emerson, O. Park, T. Park and K. P. Schmid. *Principles of Animal Ecology*. Philadelphia: Saunders, 1949.
2. Andrewartha, H. C. and L. C. Birch. *The Distribution and Abundance of Animals*. Chicago: Univ. Chicago Press, 1954.
3. Bělehrádek, J. Temperature and living matter. *Protoplasma Monographien*, vol. 8. Berlin: Borntraeger, 1935.
4. Browning, T. O. The influence of temperature on the rate of development of insects, with special reference to the eggs of *Gryllulus commodus* Walker. *Australian J. Sci. Res.*, ser. B, 5: 96–111, 1952.
5. Hodson, A. C. and M. A. Al Rawy. Temperature in relation to developmental thresholds of insects. *Proc. Xth Int. Congress Entomology*. In press.
6. Huffaker, C. B. The temperature relations of the immature stages of the malarial mosquito, *Anopheles quadrimaculatus* Say, with a comparison of the developmental power of constant and variable temperatures in insect metabolism. *Ann. Entomol. Soc. Amer.* 37: 1–27, 1944.
7. Johnson, C. G. Development, hatching and mortality of the eggs of *Cimex lectularius* L. in relation to climate, with observations on the effects of preconditioning to temperature. *Parasitology* 32: 127–173, 1940.
8. Lin, S., A. C. Hodson and A. G. Richards. An analysis of threshold temperatures for the development of *Oncopeltus* and *Tribolium* eggs. *Physiol. Zool.* 27: 287–311, 1954.

8a. Richards, A. G. and M. Q. Kolderie. Variation in weight, developmental rate, and hatchability of *Oncopeltus* eggs as a function of the mother's age. *Entomol. News* 68: 57–64, 1957.

9. Uvarov, B. P. Insects and climate. *Trans. Entomol. Soc. London*, 79: 1–247, 1931.

SOME THEORETICAL ASPECTS OF EFFECTS OF TEMPERATURE ON PLANTS

F. W. Went, *Division of Biology, California Institute of Technology, Pasadena, California*

Temperature is an excellent tool in the investigation of physiological processes in general, even though its effects are not specific since practically all processes are influenced simultaneously by temperature, and since we cannot supply the temperature only to a particular process, we are always dealing with complicated interreactions. However, in previous work it was found that usually only one of the many processes which are affected by temperature is controlling the behavior of an organism and thus the temperature effect on an organism as a whole becomes analyzable.

As a first example, the effect of temperature on the growth of tomato plants will be discussed. When tomato plants are grown under a whole range of temperatures, it is found that their growth and fruiting behavior can be correlated very strongly with the nyctotemperature which prevails during the dark period of each day (10). This is understandable since 80–90% of the growth of a tomato plant under natural conditions occurs during darkness. Thus in curve A of figure 1 this temperature relationship is shown for the intact tomato plant. It will be seen that in a mature plant there is an exponential increase in growth rate as the temperature increases. This increase, however, levels off and an optimal rate of growth is obtained at nycto-temperatures of 15°–18°, depending on the variety of tomato. Above the optimal temperature of about 17° the growth rate drops off, although rather slowly, with further increases in temperature. It is obvious that around 17° there is a complete shift of control in the growth of the tomato plant. Below 17° the temperature coefficient suggests control by a chemical process, whereas above 17° a very different process must be taking over.

In an analysis of the factors limiting tomato growth in different temperatures, it can be made likely that below 17° the actual growth process is limiting. This can be seen, for instance, in experiments where plant hormones are supplied at different temperatures (9). When emasculated flowers are treated with auxins the fruits will develop parthenocarpically. However, at high nycto-temperatures auxins have no effect on this fruit set. It is only under 17° that strong effects of the applied auxin are obtained.

It was also found in other plants that plant hormones are ineffective in increasing growth at higher temperatures (for instance, Bonner, ref. 1, with the application of thiamine to *Cosmos*).

When isolated tomato cells or tomato roots are cultivated at different temperatures, they will increase their growth rate with increasing temperature to well above 25°, which means that the low temperature optimum of the intact plant is in some way related to its being a complete organism (curve B, fig. 1). The difference between an excised root which receives its food supply from all directions in a nutrient solution and an intact root is largely that the latter has to be supplied through its conducting tissues by the top of the plant. This suggests that the low temperature optimum is in some way connected with this supply of sugar and other nutrients from the leaves where these are formed through photosynthesis. In many series of experiments it was actually found (10, 13) that the translocation

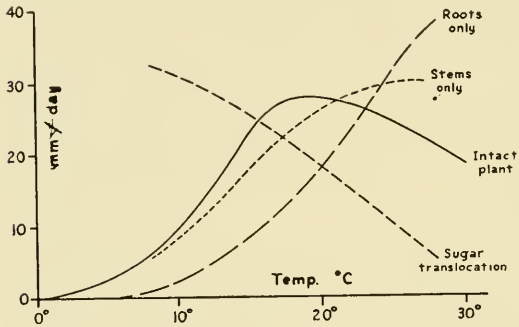


FIG. 1. Effects of nyctotemperature on growth of mature intact tomato plants (curve A), isolated tomato roots grown *in vitro* (curve B) and sugar translocation in tomato stems (curve C) From Went 1944a.

of sugar from leaves to other parts of the plant has a Q_{10} of less than 1 (see curve C, fig. 1). Therefore, as the nycto-temperature for a growing tomato plant is raised higher and higher, first the growth process is accelerated until a point is reached where the supply of sugar and other nutrients cannot keep up with the other phases of the growth process and from that point on the temperature curve of growth will follow the temperature effect on translocation. There are many facts which can be explained with this two-factor scheme. When a tomato plant has been grown for a week or longer at nycto-temperatures below 17°, the supply of sugars to the growing points has been in excess of what is being used. When afterwards for one or two nights the plants are exposed to a night temperature of 26° or 30° their growth rate is 50–100% greater than it could ever be in the steady state, but in the third or fourth night returns again to the normal rate at the new temperature. Another fact which can be explained this way is that the optimal nycto-temperature is very high for seedlings and gradually decreases to an optimum of 17°. This decrease is not a function of the age of

the plant but a function of its actual size. That is to say, that the further the growing points are removed from the leaves or supply organs, the lower the nycto-temperature is because the more translocation becomes limiting.

Not only in tomato, but in many other plants, this Q_{10} under one for sugar translocation explains observations in the laboratory and in nature. Sugar beets, for instance, attain a sugar concentration in their storage organs which is inversely proportional to the temperature, and therefore sugar beets growing in cooler climates have invariably a higher sugar content than those grown in warmer climates. For this reason also, sugar beets in the hot Imperial Valley can be grown during winter but have to be harvested before the warm weather of summer. The explanation usually given for this phenomenon, namely that at the higher temperatures the sugar is removed by excessive respiration, is invalid because respiration removes in such beets only a fraction (less than 30%) of the sugar formed

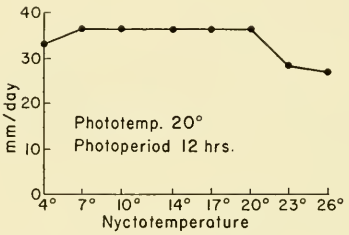


FIG. 2. Maximum growth rate of Alaska pea (ordinate) kept at different nyctotemperatures From Went 1957.

during the day in photosynthesis. Besides, it was found in tomatoes that the sugar content of leaves is higher after a warm night than after a cool one, due to the slow translocation at the high temperatures.

The effect of temperature on the growth of peas is somewhat similar to that described in tomatoes, but especially in young peas there is a considerable range of temperatures where growth is not influenced by temperature at all. Between 7° and 20° nyctotemperature (see fig. 2) the maximal growth rate of pea plants is exactly the same. This means that a diffusion process is limiting growth over that region. At lower temperatures the growth process is again limiting but at higher temperatures injurious effects cause very rapid decline in growth rate with time.

The effects of temperature on growth in general are mostly comparable with those described in the previous paragraphs. Consequently, we can draw another conclusion. Since apparently at the optimal temperatures with a Q_{10} near one we are dealing with processes limiting growth by diffusion or a similar process, it is unlikely that it would be possible to speed up growth beyond the maximum rate which can be attained at optimal temperature. It was already shown that hormones are unable to increase

growth above the optimal temperature and a better supply of nutrients is possible only at lower temperatures. Therefore it seems that we are dealing here with an absolute limit in the growth rate of plants. Unless we can influence this diffusion process by some means, the chances of extending the presently established maximum growth rate are not very good.

These diffusion processes are apparently localized in the growing points and are either inter-cellular or intra-cellular over short distances. If they are inter-cellular, a possible speed-up of this process would be by decreasing the cell size and thus increasing the diffusion rate. In this connection, it is probably significant that all fast-growing plants have relatively small cells and small nuclei, whereas the largest nuclei are usually found in plants which grow rather slowly. It is also significant that though through polyploidy it is possible to increase the size of the individual cells, usually the growth rates of these cells and the organs built out of them are decreased in comparison with the diploid situation. Octoploids always grow very much slower. This does not necessarily have to be explained on the basis of upset gene balance, but would follow from growth control by inter-cellular diffusion processes.

There are also indications that intra-cellular diffusion processes may be of significance in growth. When a number of pea seeds are segregated according to their size and grown under controlled conditions we find that the smallest peas develop into plants which originally had the highest growth rate. The larger peas, on the other hand, grow more slowly in the beginning. One to two weeks after germination this trend reverses itself so that the length of three-week-old peas is the same whether the plants came from small or big seeds. This phenomenon seems explainable on the basis that in the larger growing points of the big seeds diffusion processes leading to growth are slower than in the smaller growing points of small peas which therefore can grow faster. It is conceivable that the faster growing rate will result in larger growing points which then automatically slow down further growth. In this way an auto-regulatory feedback mechanism would exist which could control the growth of these peas within rather narrow limits.

Other processes in plants are influenced in a very different way by temperature. In the case of protoplasmic streaming there is a direct proportionality between temperature and velocity of streaming (fig. 3). Although this has been explained on the basis of several processes with opposing temperature-dependence, this explanation does not really account for the remarkably straight line temperature relationship. In the case of the morphological development of the pea plant we also find such direct proportionality between temperature and the rate of node formation. Figure 4 gives an example of this. Also, there is no indication that we are dealing

with control by a set of different factors with different temperature relationships. This is particularly clear since the same linear relationship holds between node formation and light intensity (fig. 5), or length of photo-

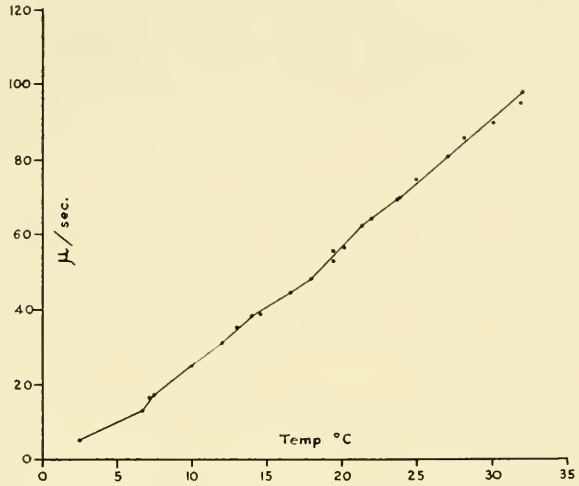


Fig. 3. Relationship between temperature and rate of protoplasmic streaming in *Chara foetida* From Hille Ris Lambers 1926.

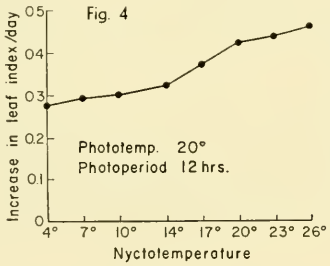


Fig. 4. Rate of node formation in stem tips of Alaska peas as a function of the nyctotemperature From Went 1957.

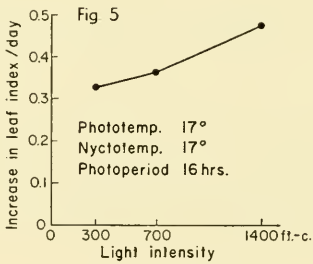


Fig. 5. Rate of node formation in stem tips of Alaska peas as a function of the light intensity under which they are grown From Went 1957.

period. Therefore we will have to find some basic process which is not influenced like a chemical reaction by temperature.

When tomato or other plants are grown in continuous light and constant temperature, their growth is soon reduced and in the case of the tomato curious injury symptoms appear partly in the form of bleached leaf areas and partly by necrotic spots on the young developing leaves (7). At

present Mr. T. Kristofferson is investigating this process in greater detail and, like Hillman, he has found that by fluctuating the light, the injury symptoms will disappear. At the same time, injury can be prevented by keeping the plants in continuous light, but subjecting them to a 24-hour cycle of temperature fluctuation. This in itself is most interesting, because here we find an equivalence of temperature and light in preventing the injury just described. When peas are grown in constant conditions no injury symptoms appear, but as Highkin has found (5), in the course of

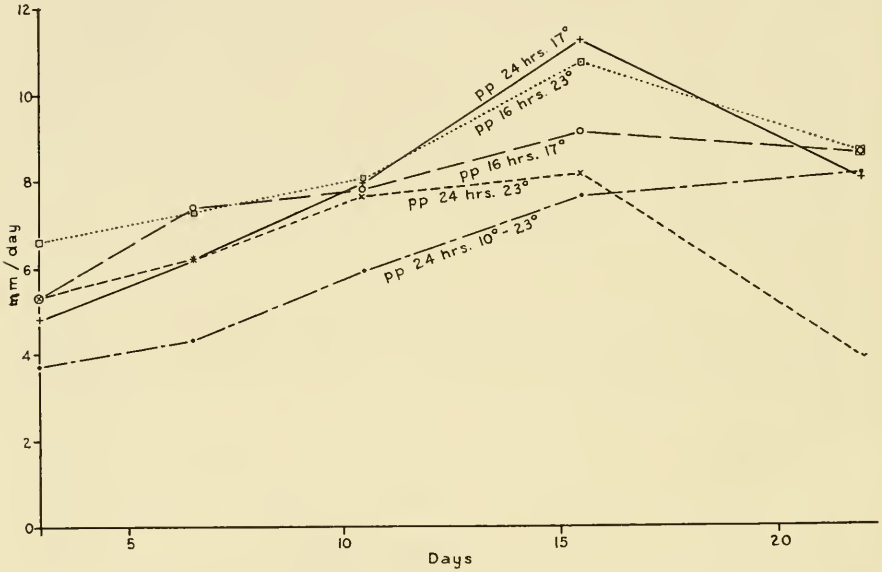


Fig. 6. Growth rate as a function of age of Zelka pea plants, when these are grown in continuous light (photoperiod, pp = 24 hr.) or in light-dark alternation (pp = 16 hr.), either in a constant temperature of 17° or 23°C or in alternating temperature (16 hr. at 10°, 8 hr. at 23°).

several generations the ability of these plants to grow is seriously impaired. When pea plants are grown for only one generation it will be found that plants in which either light or temperature is fluctuating with a 24-hour cycle continue to grow vigorously, whereas the growth of plants kept in continuous light and constant temperature decreases in the course of four weeks.

This phenomenon is shown in figure 6. Whereas in the early stages of growth the peas grew slowly, especially at the low temperatures, soon the rate increased. But in constant temperature and light, the growth rate reached a maximum rate, after which it decreased more or less rapidly. At fluctuating temperatures this drop in rate did not occur or came much later.

Another phenomenon was associated with growth under constant conditions. The variability of the peas (both Zelka and Unica varieties) was much greater, indicating that part of the regulatory mechanisms controlling growth are not operative at constant temperature or in constant light. It was found that fluctuations in temperature and light are equivalent in their effect on reducing variability, and that a 7° or 13°C temperature or an 8- or 16-hour photoperiod light fluctuation are all about equally effective (see table 1). Therefore it seems that the significant aspect is not the intensity of the fluctuating factors, but the fluctuation itself.

In addition to the requirement of fluctuating temperature, expressed in the term 'thermo-periodicity' which is apparently important in a large number of plants, there is another requirement of this periodicity, namely, it must have a 24-hour period. When the cycle is either shortened or lengthened, the fluctuation in light is unable to prevent damage due to continuous illumination. Obviously we are dealing here with the 24-hour

Table 1. Coefficients of variability of unica pea plants grown for 3 weeks under different conditions*

temperature	light	1st week	2nd and 3rd week
constant	continuous	19.6 (6)	17.0 (9)
constant	intermittent	14.5 (12)	10.7 (18)
fluctuating	continuous	15.8 (4)	10.2 (6)
fluctuating	intermittent	14.0 (9)	11.7 (17)

* In parentheses the number of groups from which the coefficients of variability were averaged.

autonomous cycle which Bünning has shown to be present in so many plant processes and which he considers to be the basis of photoperiodism. In the tomato a number of processes were found which have to have a 24-hour cycle to make the plants develop normally. For instance, when such tomato plants, instead of receiving 8 hours light and 16 darkness in each 24-hour period, are given 4 hours light and 8 hours darkness over every 12-hour period, then growth of these plants is considerably reduced, even though they receive exactly the same total amount of light. It will be seen that in such plants at the beginning of each light period the leaves are in so-called night position and therefore photosynthesis during the 4 hours would have to occur largely in the scotophil-phase of Bünning.

Bünning in earlier work (2) had found that this autonomous rhythm of the nyctinastic movements of bean plants had a slight temperature-dependence although more recently one of his students (8) could not confirm this temperature-dependence. On the basis of a slight increase in cycle length with a decrease in temperature it is possible to explain the behavior of a number of tropical plants under cold growing temperatures.

When either African violets or begonias are grown on a 24-hour light cycle (for instance, 8 hours light and 16 hours darkness) then in the course of several weeks or months they will die at 10°C, although at 23° they develop normally and flower abundantly. When, at 10°, they are grown in a 32-hour cycle of 10.7 hours of light and 21.3 hours of darkness, they will develop well, although slowly, and form strong dark-green plants (fig. 7). Their failure to grow at 10° in a 24-hour cycle of light-darkness is obviously due to a lack of synchronization of their internal autonomous Bünning-cycle, which at that low temperature is significantly longer than 24 hours, and the external 24-hour cycle. This explains also why their

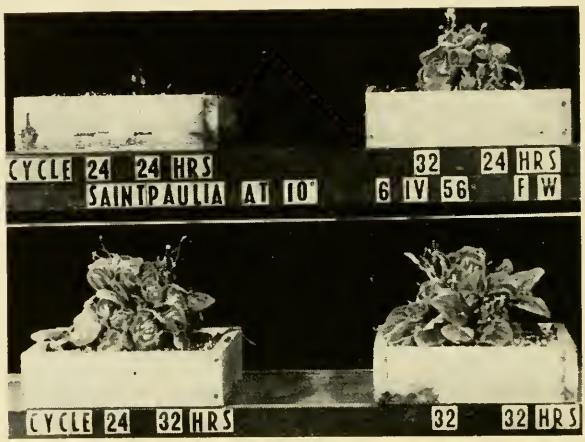


Fig. 7. Saintpaulia plants, all grown in a constant temperature of 10°C. *Upper left:* plant kept for 5 months on a 24-hr. light-dark cycle; *lower right*, plant kept on 32-hr. cycle; *upper right*, first 2 months on 32-hr., last 3 months on 24-hr. cycle; *lower left*, first on 24-hr., then on 32-hr. cycle.

death at 10° is so slow: no toxic materials are formed, but further development of the growing points has become impossible, and the previously nitiated leaves remain small and chlorotic.

It is likely that many other tropical plants which cannot grow at the lower temperatures of temperate climates, even though the temperature remains above the freezing point at all times, die when their Bünning cycle is sufficiently slowed down by low temperatures so that they get out of phase with the 24-hour day. Similarly, the actual dying of cool climate plants at higher temperatures (like *Baeria chrysostoma* which dies at 26°C nycto-temperature) might be attributable to a speed-up of their 24-hour cycle at the higher temperatures.

How can we imagine that thermoperiodicity influences development especially if the periodic fluctuations in temperature of a 24-hour period can

be replaced by light fluctuations? For this we must try to find a cyclic process in the development of a plant, which could be influenced by external cycles. Such processes actually have been known for some considerable time. It has been common knowledge among cytologists that at certain hours of the day (or especially of the night) the number of mitoses is much greater than at other times. This has recently been re-investigated by Bünning (3) who found that in stem tips most cell divisions occur during a few night hours (see fig. 8). For roots no such periodicity in mitoses was found. Bünning was unable to correlate this with the photoperiodic behavior of these plants (*Perilla* is a short-day plant, *Spinacia* a long-day).

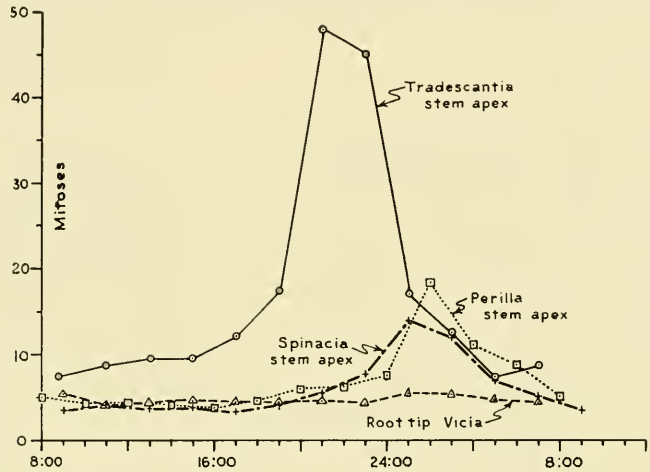


FIG. 8. Number of mitoses at different times of day and night, either in roots of *Vicia faba* (no periodicity) or in apical meristems of *Tradescantia, Spinacia* or *Perilla* From Bünning 1952.

That is to say, the mitosis maximum occurred at the same time for both long- and short-day plants.

It is obvious that in the growing point of the stem tip of a plant there must be a perfect regulation of cell divisions to produce the exactly spaced organs. This could conceivably be a diffusing substance or hormone. But no such hormones have been found. Besides, we do not know of any radiation which induces mitosis. But the mitoses in the stem tip are perfectly synchronized with a certain night hour. Therefore the assumption is obvious that the cells in the stem tip have a certain period over which they can divide, which period becomes synchronized through a 24-hour cycle in temperature, or light, or both. If we assume, for instance, that 48 hours after one division the cell is again able to divide, a plant kept on a 36-hour cycle would have perhaps some of its cells divide after 36 hours, the others

after 72 hours, whereas in a 24-hour cycle they presumably would all divide after twice 24 hours. Therefore any departure from the normal 24-hour cycle, or from the length of cycle induced by abnormal temperatures, will disrupt this synchronization of cell divisions in the growing point and cause irregularities in growth. In this way thermoperiodicity has a function like hormones in the coordination of growth. These considerations approach rather closely the work reported recently on synchronization of cell divisions in microorganisms, where discreet changes in conditions may cause all cells to divide simultaneously. This induced rhythm will gradually be lost, unless new inducing conditions are applied.

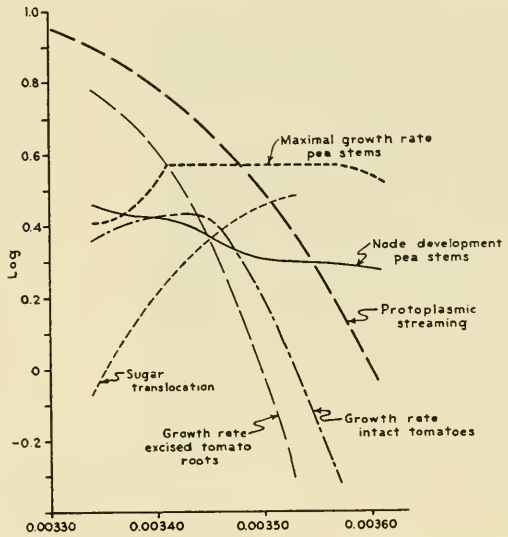


FIG. 9. Arrhenius plots of temperature dependence of various processes, presented in figs 1, 2, 3 and 4. *Abscissa:* inverse of absolute temperature; *ordinate:* logarithm of the rate of the process.

As a historical note it can be mentioned that in the time of de Candolle 100 years ago, temperature was considered a major factor in the effect of climate on vegetation. It is rather remarkable that the primitive concept of de Candolle (4) has not undergone any development in connection with newer knowledge, but that among agroclimatologists the 'heat sum' is still commonly used. This concept presupposes a direct proportionality between temperature and development of a crop plant, more or less like the effect of temperature on protoplasmic streaming. From figures 1 and 2 it can be seen that only over a very short range, growth of tomatoes (and other plants) is more or less proportional with temperature. Therefore a 'heat sum' is impermissible since it adds all effects of temperature, whereas above the optimal temperature these effects rather should be subtracted. Or, more accurately, the temperature curve of most plants can be represented by a cubic equation.

For many chemical reactions, and sometimes complex biological processes as a whole, a plot of the logarithm of the net reaction rate against the reciprocal of the absolute temperature gives a straight line relationship, in accordance with the Arrhenius theory of chemical reaction rates. But when all the plant reactions described in the preceding paragraphs are plotted in this way, the deviations from a linear relationship show that these complex processes are subject to control by a number of reactions which cannot be analyzed as a whole on the basis of the Arrhenius equation (fig. 9). Certain deviations of complicated biological rates away from the Arrhenius equation have been interpreted quantitatively with some success. The present paper suggests the manner in which other factors are involved in complicating any simple relationship for the variation of plant processes with temperature.

SUMMARY

Effects of temperature on plants are manifold. In some instances temperature clearly influences the activation of a chemical process, in which the Q_{10} ranges between 2 and 3, as most clearly expressed in the effects of temperature on respiration. Above 35°C the rate of respiration is decreased with a pronounced time factor, suggesting a superimposed temperature effect on protein denaturation. For many individual physiological processes such exponential relationships have been found. But for an even greater number of processes, especially of intact organisms, different and far more complicated temperature relationships exist.

The effect of temperature on protoplasmic streaming is apparently simple. When the rate of streaming is plotted against temperature an almost straight line appears. This means that the Q_{10} steadily decreases from a high value to well below 2. Here an exponential plot obscures the real relationship. The same straight line temperature relationship exists for the effect of temperature on morphological differentiation in the growing point of peas, for instance.

For stem elongation and dry weight production in intact plants a very different temperature relationship exists. According to the species, variety, age and size of plant a different, but usually low, optimal temperature is found. In the tomato plant the optimal temperature in light is 23°C; in darkness it is high (above 25°C) in the seedling stage, and decreases gradually to 15°–18°C in mature plants, at least in full daylight. At lower light intensities this night optimal temperature drops to below 10°C. Greenhouse tomato varieties have lower optimal temperatures than outdoor varieties. Thus far, breeding has not extended the temperature range of tomato varieties, and they still require a relatively warm climate.

Again other processes have a temperature coefficient only slightly above

1. The growth in length of young pea plants is independent of temperature over the range of 13°–23°C. The internal 24-hour rhythm of development of most plants is almost temperature independent.

At a Q_{10} of 2–3 it can be assumed that a chemical process controls plant growth. Below 17°C it can be reasonably certain that reactions involving auxin and other growth substances limit growth: only below 17° can tomato growth be accelerated by auxin or vitamins. Above this temperature physical processes involving all molecules, and not just the activated ones, become limiting for plant development.

REFERENCES

1. Bonner, J. Effects of application of thiamine to *Cosmos*. *Bot. Gaz.* 104: 475–479, 1943.
2. Bünning, E. Untersuchungen über die autonomen tagesperiodischen Bewegungen der Primärblätter von *Phaseolus multiflorus*. *Jahrb. wiss. Bot.* 75: 439–480, 1931.
3. Bünning, E. Über den Tagesrhythmus der Mitosehäufigkeit in Pflanzen. *Zeit. f. Bot.* 40: 193–199, 1952.
4. Candolle, A. de Géographie botanique raisonnée I. Paris: Masson, 1855, 606 pp.
5. Highkin, H. *Plant Physiology*. Supp., Aug. 26, 1956, vol. 31, p. iii.
6. Hille Ris Lambers, M. *Temperatuur en protoplasma-strooming*. Thesis, Utrecht, 1926.
7. Hillman, W. S. Injury of tomato plants by continuous light and unfavorable photoperiodic cycles. *Am. J. Bot.* 43: 89–96, 1956.
8. Leinweber, F. J. Über die Temperaturabhängigkeit der Periodenlänge bei der endogenen Tagesrhythmik von *Phaseolus*. *Zeit. f. Bot.* 44: 337–364, 1956.
9. Osborne, D. J. and F. W. Went. Climatic factors influencing parthenocarpy and normal fruit set in tomatoes. *Bot. Gaz.* 114: 312–321, 1953.
10. Went, F. W. Plant growth under controlled conditions. II. Thermoperiodicity in growth and fruiting of the tomato. *Am. J. Bot.* 31: 135–140, 1944.
11. Went, F. W. Plant growth under controlled conditions. III. Correlation between various physiological processes and growth in the tomato plant. *Am. J. Bot.* 31: 597–618, 1944a.
12. Went, F. W. The experimental control of plant growth. *Chronica Botanica* 17: 1–343, 1957.
13. Went, F. W. and H. Hull. The effect of temperature upon translocation of carbohydrates in the tomato plant. *Plant Physiol.* 24: 505–526, 1949.

MODIFICATION BY LOW TEMPERATURE OF CERTAIN ION RESPONSES OF BULLFROG NERVE FIBERS[1]

FREDERICK CRESCITELLI, *Department of Zoology*
University of California, Los Angeles, California

THIS COMMUNICATION describes a number of responses to sodium and to potassium ions by the A and B fibers of the bullfrog sciatic-peroneal nerve at normal and at low temperatures. The results demonstrate some notable differences in these two fiber groups with regard to their reactions to low temperature. This study is part of a larger program, from which some data have already appeared (5, 6), whose aim is to point out and to analyze differences in properties of different fiber groups in vertebrate peripheral nerves. In this introduction a review or analysis of the literature concerned with the effects of temperature on nerve fibers will not be attempted. Some summaries and enumeration of existing information have appeared in the publications of Lorente de Nó (15), Hodgkin and Katz (13), Hertz (11) and others. References to relevant literature will be made in appropriate places in the body of this report.

DIFFERENTIAL SENSITIVITY TO SODIUM AND TO POTASSIUM AT NORMAL TEMPERATURE

Following appropriate stimulation, the sciatic nerve of the bullfrog *(Rana catesbiana)* yields a compound electrogram with three distinct elevations known respectively as the A, B and C waves or action potentials (fig. 1). These are known to represent activity in three different groups of fibers each with characteristic physiological and pharmacological properties. At normal temperatures the A, B and C fiber groups react to solutions with heightened potassium by a characteristic differential block of conduction at the treated portion of the nerve. These same fibers are also blocked selectively in response to solutions lacking sodium but this differential block is qualitatively different from the high potassium action. These points are illustrated by an experiment (fig. 1) which involved the recording with a dual beam cathode ray oscillograph of the A, B and C action potentials. The lower beam pictures the actions of all three fiber groups while the upper trace shows, especially amplified, the B and C activity. Record 1 is a control monophasic electrogram taken just before

[1] Aided by a grant from the Division of Research Grants and Fellowships, National Institutes of Health, U.S. Public Health Service, and by a research grant from the Board of Research, University of California.

addition of the test solution. The test solution, containing all the constituents of Ringer's fluid at their usual concentrations except for the KCl, whose concentration was raised 10 fold, i.e. to 18 mM, was then added to a 20-mm nerve segment located between the stimulating and recording

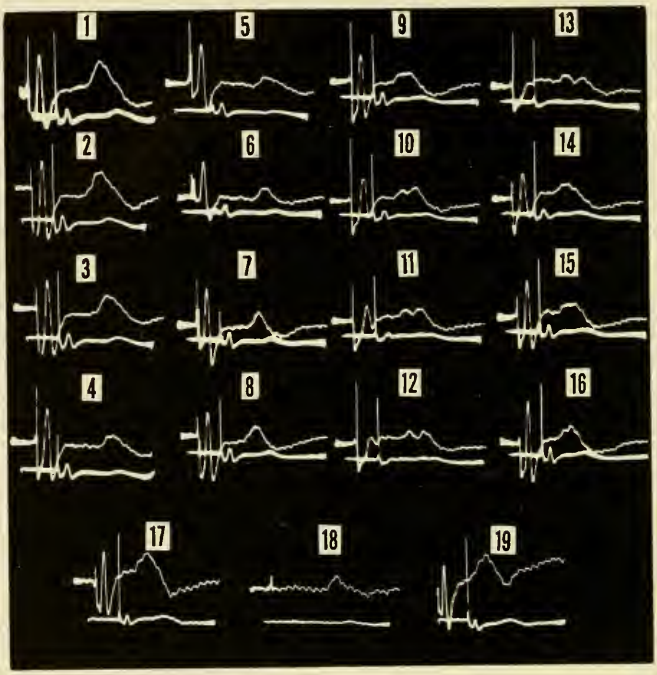


FIG. 1. Selective responses to potassium and sodium. Dual beam recording with B and C fibers on upper beam and A, B and C fibers on lower beam. Record 1 is control; records 2 to 6 show action of a solution with a concentration of 18 mM KCl. Records were made at 5′ (2), 7′ (3), 11′ (4), 16′ (5), and 60′ (6) after adding the test solution. Records 7–13 show action of a solution with limiting concentration (11 mM), of NaCl. Records were taken at 1′(7), 2′(8), 5′(9), 13′(10), 18′(11), 23′(12) and 31′(13) after the test solution. Records 14–16 picture the recovery in Ringer's solution. Records were made at 30″ (14), 1′ (15), and 9′ (16) after adding the Ringer's solution. Records 17–19 show persistence of many C fibers after adding a solution with 21.6 mM KCl. Record 17 is the control; record 18 was made 20′ after adding the test solution and record 19 shows the recovery after Ringer's fluid.

electrodes. Records 2–6 illustrate the selective aspect of the potassium effect. The A fibers and many of the C fibers were especially sensitive and ceased activity in response to the test solution. In contrast, most of the B fibers and many C fibers were still conducting 60 minutes (record 6) after treatment with the test solution. This section of the experiment (records 1–6) reveals the relatively great sensitivity of the A fibers and of many of the C fibers to high potassium and, at the same time, the much lower sen-

sitivity of most of the B and some of the C fibers. The differential sensitivity to low sodium is pictured in the next section of the experiment (records 7–13). Eight minutes after electrogram 6 was recorded a solution which was deficient in sodium was added to the nerve segment. This solution had the normal amount of potassium but the NaCl concentration was reduced to one-tenth that of Ringer's solution. Choline chloride was employed to replace the missing NaCl so as to obtain osmotic equivalence. Record 7, obtained 1 minute after this addition, shows the rapid recovery of many A fibers and of some of the C fibers. Two minutes after the addition (record 8) a majority of the A fibers and a few more C fibers had recovered from the potassium block. While A fiber activity was being restored and maintained (records 7–13), the B group underwent a gradual reduction and finally ceased activity (record 13). In other words the solution with 11 mM NaCl was able to support most of the A but none of the B activity. The solution with 11 mM NaCl caused the C wave to change in form and to assume a double-humped configuration (records 10–13). The changes in the C potential were such as to suggest the existence of two types of C fibers: one group relatively sensitive to high potassium but relatively resistant to the low sodium and a second group with the inverse relation to these two cations. The B and the potassium-resistant C fibers were not equally resistant to high potassium. Through the use of a carefully selected concentration of potassium ions it was possible to demonstrate that these C fibers would even outlast the B group. This point is illustrated in records 17–19 (fig. 1). Record 17 is the control. A solution with KCl at 12 times the Ringer concentration was then added and record 18, obtained 20 minutes later, depicts the complete cessation of activity in the A (only shock artifact remains), the B and many of the C fibers. A significant number of C fibers remained in action, however, even after all B activity had ceased. The addition of Ringer's solution caused complete recovery in all the blocked fibers (record 19).

Another type of experiment (fig. 2) shows not only the differences in responses of the A and B fibers to sodium and to potassium but also some interesting additional features in the actions of these two ions. The experiment was designed so that sodium and potassium were added simultaneously to the blocked nerve segment and could act competitively, the one (sodium) to produce recovery and the other (potassium) to produce secondary block. The experiment consisted of seven different steps all carried out on the same nerve preparation. For each of these steps the nerve segment was first exposed to a solution lacking NaCl and KCl. Tetramethylammonium bromide (TMA) was employed as the osmotic substitute. Total conduction block, due to sodium lack, occurred in each of these seven steps but this is not included in figure 2. The figure presents only the 'recovery' curves which resulted in each of these seven procedures

when the blocked nerve segment was treated with solutions all with KCl at five times the Ringer level but with the NaCl varied in the seven steps from 110 mM to 5.5 mM. It is convenient to consider these 'recovery' curves in terms of an initial, rapid restoration, due to sodium, and a phase of secondary block due to potassium. If the initial phase is considered, one sees again the low sodium differentiation between the A and B groups of fibers. Thus a concentration of 11 mM NaCl caused initial recovery in the A but not in the B action potentials. It is evident that the presence of potassium at five times the normal concentration did not alter this

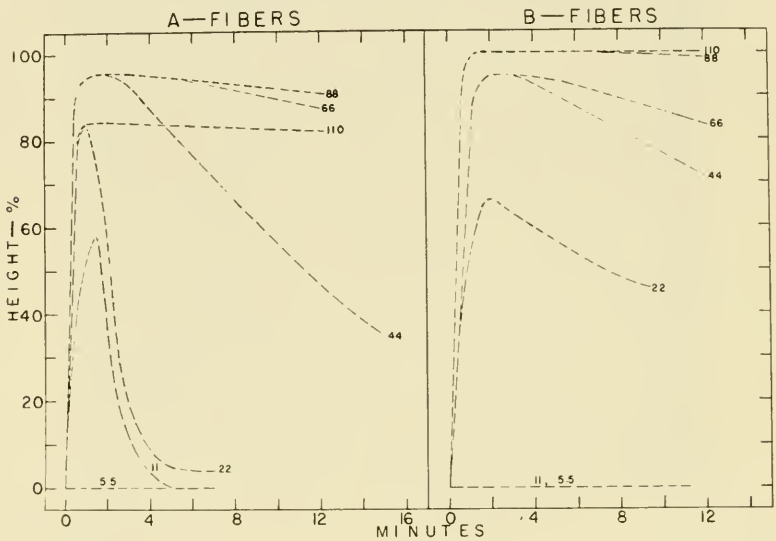


Fig. 2. 'Recovery' of A and B fibers following block by a solution lacking NaCl. Solutions for 'recovery' all contained 9.0 mM KCl and varying concentrations of NaCl as indicated (in mM) with each individual graph. Tetramethylammonium bromide was employed as the osmotic substitute for the deficient NaCl.

selective effect of sodium ions. If the secondary phase is considered one sees also the selective behavior in response to high potassium. Thus when secondary block occurred at all it was prominent in the A fibers but absent or small in the B group. These curves also demonstrate that secondary block with a constant level of potassium was, in addition, related to sodium concentration. Thus with a sodium level of 110 mM no secondary block was recorded for the A fibers. As the sodium concentration was decreased below this value the rate of secondary block of the A fibers was markedly enhanced. This effect may have been the result of an increased rate of depolarization by potassium ions in the presence of a deficiency of sodium. Lundberg (19) has reported that certain cations can modify the potassium

depolarization of bullfrog nerve fibers. While experiments with sodium-deficient solutions were not listed by Lundberg he did include experiments with sodium at twice the normal concentration which were interpreted as indicating an inhibition of the potassium depolarization. He also reported that the conduction block by excess potassium could be greatly delayed by increasing the sodium concentration. There is one great difficulty in utilizing Lundberg's data in an interpretation of the accelerated potassium block in the presence of deficient sodium (fig. 2). TMA was employed as an osmotic substitute in the experiments of figure 2. Lundberg reported that this organic cation was capable of inhibiting posassium depolorization to a degree even greater than sodium. In terms of Lundberg's results the combination of TMA and sodium in the present experiments should have been at least as equally effective as 110 mM NaCl in inhibiting depolarization by potassium and slowing down the rate of block. Since this was not the outcome of these experiments there is either some unaccountable factor in one of these investigations or else a simple explanation in terms of depolarization is insufficient at these low sodium levels. The experiment of figure 2 also suggests a rather interesting difference in temporal action on the part of sodium and potassium ions. This is especially well shown for the A fibers in the step which employed NaCl at a concentration of 11 mM. This solution, though unable to maintain activity in any of the A fibers, was nevertheless able to bring about a significant transient recovery in many of them. The action of potasium in causing secondary block must have been delayed in relation to the action of sodium in relieving the low sodium block.

ACTION POTENTIALS AT LOW TEMPERATURE

When an isolated nerve preparation, mounted on its electrodes within a moist air chamber, was transferred from a normal temperature of about 23°C to a low temperature (5°C or less) a progressive change in height of the compound spike was recorded. The detailed changes were slightly different, in a quantitative sense, for different nerve preparations but the important features are adequately emphasized in figure 3. The data obtained with the sheathed nerve (fig. 3) will serve to illustrate the procedure which was followed. The nerve, after dissection, was placed in position on the electrodes in the moist chamber in a constant temperature water bath at 23.5°C. After allowing a period for equilibration the monophasic A compound spike was recorded employing as a stimulus a shock at three times the strength required to elicit maximal A activity. The nerve within its chamber was next transferred to a second water bath operating at 2.2°C. The first record obtained in about a minute after this transfer was almost identical in height to the response obtained at 23.5°C.

The height of this first electrogram was arbitrarily assigned the value 100 and subsequent records were all expressed in terms of this initial height. It is clear (fig. 3) that the action potential increased with time and then suffered a decline. The finding of an increase in spike height as the nerve cooled is not surprising in view of previous reports (13, 22) which demonstrated for single fibers an increase in spike height and a large in-

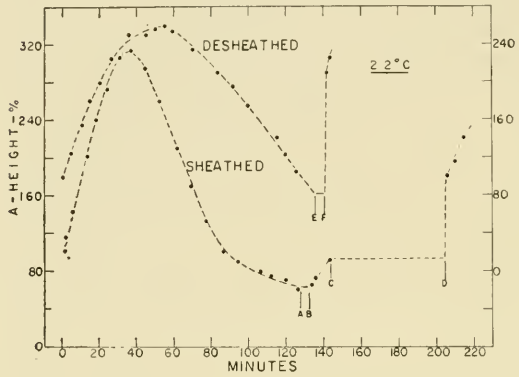


Fig. 3. Effect of low temperature (2.2°C) on height of A wave. Spike height (in percentage of initial height) plotted as a function of time (min.) after transfer of nerve to low temperature bath. Periods E–F, A–B and C–D represent wash-out periods as described in text. Ordinate at right (0–240) applies to curve for desheathed nerve; ordinate at left (0–320) to curve for sheathed nerve.

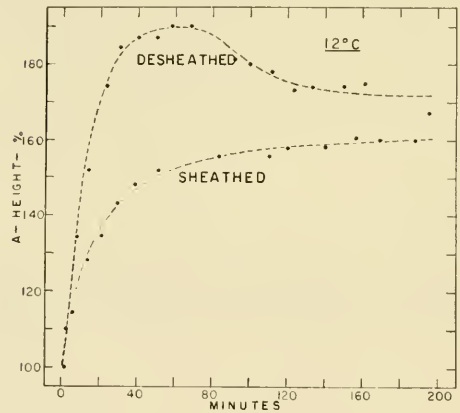


Fig. 4. An experiment similar to that in fig. 1 but with a temperature of 12°C.

crease in spike area. The interesting feature of these results is that the height, instead of remaining constant after the initial increase, declined progressively and in some experiments even fell below the 100% value. These changes were observed to occur with desheathed as well as with sheathed nerve preparations (fig. 3). No extensive study was made of the matter but the B fibers behaved qualitatively in the same fashion in response to low temperature. The C fibers were unable to survive the cold and will not be considered in this discussion.

The secondary decline in the A spike was associated with the low tem-

perature and was not the result of some adverse condition in the procedure other than low temperature. This conclusion is suggested by the results of a procedure (fig. 4) performed, not at the low temperature, but at a so-called intermediate temperature. The outcome of this experiment, carried out at 12°C, showed the initial increment but the secondary decline was absent. Exposure to low temperature for a relatively long period was apparently the requirement for eliciting the secondary decrease.

THE WASH-OUT EFFECT

In the course of the investigation a finding was encountered which throws some light on the nature of the secondary decline in action potential. This finding, named the wash-out effect, is summarized in its essentials in figure 3. It will be described first of all for the desheathed nerve. At the moment when the A spike had declined to the 80% level, the entire nerve was lifted off the electrode system and immersed in a large volume of Ringer's solution at 2.2°C. The nerve remained in this cold solution for 5 minutes (E to F) following which it was quickly replaced on the electrodes. Records taken soon after this replacement showed that the A potential had been restored nearly completely (fig. 3). Thus a wash-out of the nerve without change in temperature was alone required to reverse the secondary decrease. The wash-out effect was also demonstrable in nerves with intact sheaths but in these cases a much longer period of wash-out was necessary to reverse the secondary block. This point is brought out by the lower graph in figure 3. In this case a 5-minute wash-out in cold Ringer's fluid (A to B) caused relatively little recovery of the action potential whereas a wash-out of 61 minutes (C to D) led to a considerable recovery of activity. These results are explicable in terms of the leak, as a result of cold, of a substance (or substances) which, acting on the nerve membrane, led to the secondary decline. The wash-out procedure, presumably, flushed away the active substance and caused recovery in many of the fibers. The wash-out was less effective in the sheathed preparation because, presumably, the sheath slowed the outward diffusion of the active substance and made more difficult the procedure of washing it away from the membrane.

The nature of the active substance (or substances) is unknown but there is good reason to expect that potassium is involved. In conformity with the behavior of other cells, nerves fibers lose extra potassium as the result of low temperature (24, 25). A leak of potassium followed by accumulation at the outer surface might account either entirely or in part for the secondary decline. The wash-out effect is reminiscent of the K-effect of Feng, Hsu and Liu (9). These investigators reported that frog nerves made anoxic by treatment with nitrogen gas would stop conducting but

that activity could be restored by the simple maneuver of washing out the nerve with oxygen-free Ringer's solution. Feng, Hsu and Liu concluded that recovery was simply the result of washing away the potassium which leaked out of the nerve fibers and accumulated during the period of anoxia. A similar experiment and conclusion was published by Shanes (23) for the case of squid and crab nerve fibers.

RESPONSES TO POTASSIUM—A FIBERS

Whatever is the exact nature of the secondary decline in action potential, it is clear that low temperature experiments were complicated by

Table 1. Action of high potassium on A fibers

NORMAL TEMPERATURES						LOW TEMPERATURES					
Nerve* No.	Normal Temp. °C.	Minutes to block to			Recovery to 50%	Low Temp. °C.	Minutes to block to			Recovery to 50%	KCl† Conc.
		25%	50%	75%			25%	50%	75%		
1	21.2a	4.3	6.5	10.7	0.8	4.0b	3.7	5.0	7.0	2.1	8.0
1	23.0c	9.8	16.0	24.0	0.8						8.0
2	23.3b	10.0	16.0	23.2	0.8	4.4a	2.0	3.3	5.2	1.5	8.0
2						4.3c	1.5	2.3	3.4	2.6	8.0
3	21.3b	3.7	6.3	14.3	0.2	5.0a	0.2	0.6	1.2	2.0	8.5
3	22.0d	3.7	5.7	8.9	0.8	5.7c	1.1	2.0	2.9	1.1	8.5
4	23.0b	7.8	13.2	18.6	0.8	1.4a	1.0	2.0	2.8	2.9	8.5
4	22.7d	7.2	11.0	15.7	0.2	1.1c	1.4	2.6	3.8	3.2	8.5
5	22.5b	7.2	12.3	21.7	0.8	3.2a	1.9	3.3	5.6	3.9	8.5
5	22.5d	4.1	6.1	7.9	0.3	0.6c	1.1	2.1	3.7	7.6	8.5
6	22.2b	2.5	3.9	5.8	1.1	3.2a	0.7	1.2	2.1	5.5	9.0

* The letters a, b, c, d indicate the sequence in which the different stages were carried out.

† Relative to the KCl concentration present in Ringer's solution.

the fact that activity was not constant with time. Because of this complication experiments were designed which required only relatively short periods of time (less that 20 minutes) and a condition of acceptance of results was the occurrence of at least 80% recovery of spike height after washing with Ringer's solution. These conditions meant that only desheathed nerves could be employed since similar experiments with sheathed preparations required several hours for completion.

Low temperature caused a very marked acceleration in the rate of block of A fibers by Ringer's solution containing excess potassium, i.e. eight to nine times the normal KCl of Ringer's fluid. In six experiments, for example (table 1), the time to reduce the A wave to 50% of its initial value (value at time of addition of test solution) was, at normal tem-

peratures, 3.9–16.0 minutes with a mean of 9.7 minutes. At the low temperatures the time to 50% block averaged 2.4 minutes and the range was 0.6–5.0 minutes. Recovery of conduction through the treated segment after the addition of Ringer's solution was faster at the normal temperatures. In the same six experiments (table 1) recovery of the A spike to the 50% level required an average time of 40 seconds at the normal temperature and the range was 12–66 seconds. At the low temperatures the average recovery time was 194 seconds and the range was 66–456 seconds.

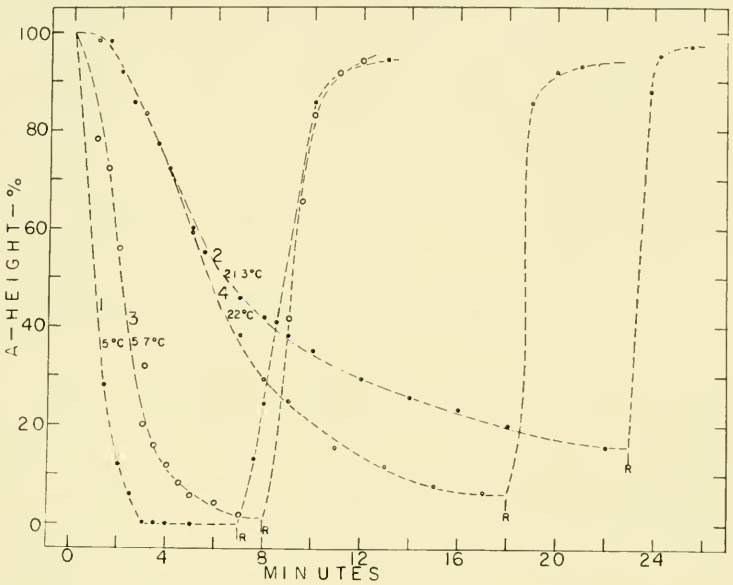


FIG. 5. Conduction block of A fibers produced by excess potassium, i.e. 15.3 mM KCl. Height of A wave (in percentage) plotted against time (min.). The four steps were carried out consecutively as numbered using the same desheathed nerve preparation. Temperatures were as indicated on graph and washing out with Ringer's solution is indicated at points shown by R.

A typical experiment and the manner in which it was carried out are illustrated in figure 5. Curve 1 depicts the course of block and recovery of the A potential at 5°C. The test solution, containing all the constituents of Ringer's fluid except for KCl which was at 8.5 times the normal concentration, was added at zero time to a 20 mm segment of the nerve between the central and peripheral portions of the nerve trunk. The central end was stimulated using a shock at 14 times the strength of the stimulus needed to elicit maximal A activity. Complete block occurred in 3 minutes and at 7 minutes Ringer's solution at 5°C was added to the nerve segment in place of the test fluid. Recovery to well over the 90% level followed. The

nerve still within its moist chamber was then transferred to a second water bath at 21.3°C. After equilibration at this new temperature the potassium treatment was repeated with the results pictured in curve 2. The nerve was then returned to the cold bath at 5.7°C and after equilibration the procedure was once more applied with the result shown in curve 3. The final step (curve 4) was a repetition of the procedure at 22°C. Taking into account all the experiments in which the order of carrying out the separate steps was varied (table 1), it is clear that progressive changes in the state of the nerve or the previous history of the preparation were not involved in the results. The data demonstrate the occurrence of a significant increase in the rate of block of the A fibers by means of a solution containing an excess of potassium and acting at low temperatures.

In view of the possibility of extra loss of intracellular potassium during the experiments at low temperatures, it was desirable to know if, and how much, of the acceleration in rate of block by cold was the result of a potassium leak. For this reason an experiment was designed in order to minimize the accumulation of substances around the nerve fibers during exposure to cold. A total of five such experiments was completed and every one led to the same conclusion. The procedure and a typical result are given in figure 6. With the nerve suspended on the electrodes in the moist chamber as for the previous experiment (fig. 5), the blocking action of a solution with KCl at six times the Ringer concentration was first tested at 23.8°C. At this temperature this concentration of KCl led to only a small reduction of the A activity (curve 1). The entire nerve was then placed in a large volume of Ringer's solution at 1.3°C. It was left in this solution for 45 minutes with frequent stirring of the fluid. The nerve was next transferred to a second large volume of physiological solution also at 1.3°C and kept in this for 5 minutes. In this manner the nerve, before recording, was equilibrated at the low temperature in a large volume of frequently stirred solution so as to provide every opportunity for the dilution and removal of substances which might have leaked out of the fibers as a result of the cold. Following this equilibration the nerve was quickly returned to the electrodes; all adjustments were made and in 4 minutes the experiment was begun. The result of the addition of the test solution with KCl at six times the normal level is presented as curve 2. In spite of all the precautions which were taken to prevent the accumulation of active substances, the low temperature still caused a great increase in rate of block. The nerve was next immersed in Ringer's solution at room temperature for 65 minutes. It was then set up once more in the moist chamber at 1.3°C and left on the electrodes at this temperature for 54 minutes. Presumably, this procedure would allow for accumulation of substances around the nerve fibers. The action of the test solution was again ex-

amined with the outcome shown in curve 3. The rate of block was greater in this third section than in the second section of the series leading to the thought that substances which leak out and accumulate may contribute a component to the result. The fourth and fifth sections were carried out in order to remove the possibility of an effect due to progressive changes in the condition of the preparation. Following the third section, just described, the nerve was placed directly into Ringer's fluid

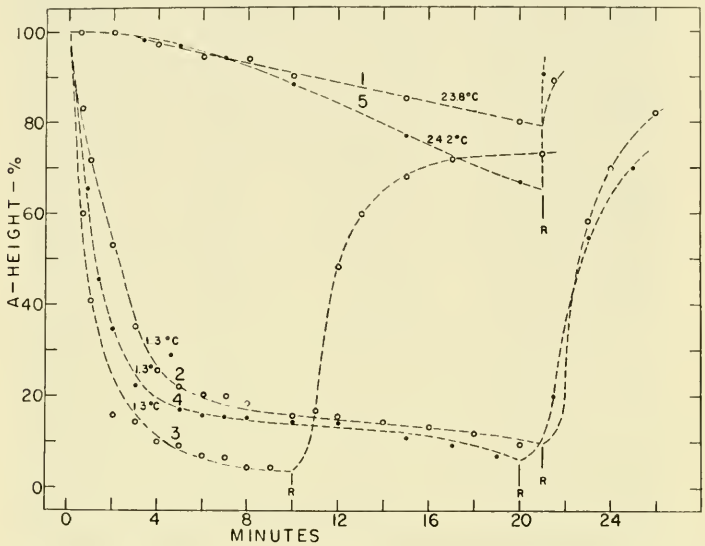


Fig. 6. Conduction block of A fibers produced by excess potassium, i.e. 10.8 mM KCl. Curve 1: block and recovery at 23.8°C; nerve on electrodes in air. Curve 2: block and recovery at 1.3°C after period of equilibration in cold Ringer's solution. Curve 3: block and recovery at 1.3°C; nerve on electrodes in air. Curve 4: block and recovery at 1.3°C with preliminary wash-out. Curve 5: block and recovery at 24.2°C; nerve on electrodes in air.

at 1.3°C and left there for 15 minutes during which time the fluid was frequently agitated. After this washing the nerve was returned to the electrodes and the potassium block was once more recorded (curve 4). The result reinforces the suspicion that, though the accumulation of an active substance made a contribution to the block, there was in addition a main effect of temperature itself on the nerve fiber which resulted in an acceleration of block by potassium ions. The fifth and final segment of the series (curve 5) simply illustrates the point that the normal temperature response was still obtainable from a nerve preparation which had suffered a rather rigorous series of low temperature treatments.

In attempting to interpret the action of cold in accelerating the potassium block the first thought which comes to mind is that low temperature enhanced the rate of depolarization by potassium ions. There is, in fact, some support for this interpretation. In a previous publication (7) the author pointed out the observation that the rate of depolarization by a solution containing 11 mM KCl was greater at 5.2°C than at 23.6°C. Lundberg (20), too, presented some evidence that low concentrations of potassium, such as those of the present investigation, caused a depolarization which was initially faster at 7°C than at 20°C. Curiously enough, this action of cold was found when the nerve was in an atmosphere of oxygen but not in an atmosphere of 95% O_2 and 5% CO_2. With higher concentrations of potassium Lundberg was unable to find any influence of temperature on the rate of depolarization. In addition there is the statement by Feng and Liu (8) "that as a first approximation the rate of potassium depolarization in nerve may be regarded as practically independent of temperature". The status of this question is therefore still somewhat uncertain and must await the elucidation of more of the factors which are involved. There appears to be enough evidence, however, to warrant the tentative conclusion that acceleration of potassium block by cold is directly related to the depolarizing action of potassium. This conclusion does not rule out, of course, the possibility that other factors may be involved; for example, that the level of depolarization which is required to produce block varies with temperature. It is interesting to note at this point that depolarizations by different substances are influenced quite differently by cold. In addition to the types of actions already mentioned for potassium there are well authenticated examples of inhibition of depolarization by a reduction in temperature. The author (7) provided an example of this in the case of amyl carbamate which, in certain concentrations, caused a progressive depolarization, an effect which was effectively abolished by cold. The depolarization associated with veratrine was found to be similarly inhibited by cold (20). The ions of ammonium and lithium apparently differ from that of potassium in that their depolarizations are delayed by low temperature (20). The nerve membrane potential can apparently be reduced by a number of actions.

RESPONSES TO POTASSIUM—B FIBERS

It has already been pointed out that the A group of fibers is more readily blocked by solutions with excess potassium than is the group of B fibers. This fact was noted by Lorente de Nó (15) for the bullfrog sciatic nerve. Differential block by excess potassium was also noted in different groups of mammalian nerve fibers by Lundberg (21). The differential sensitivity

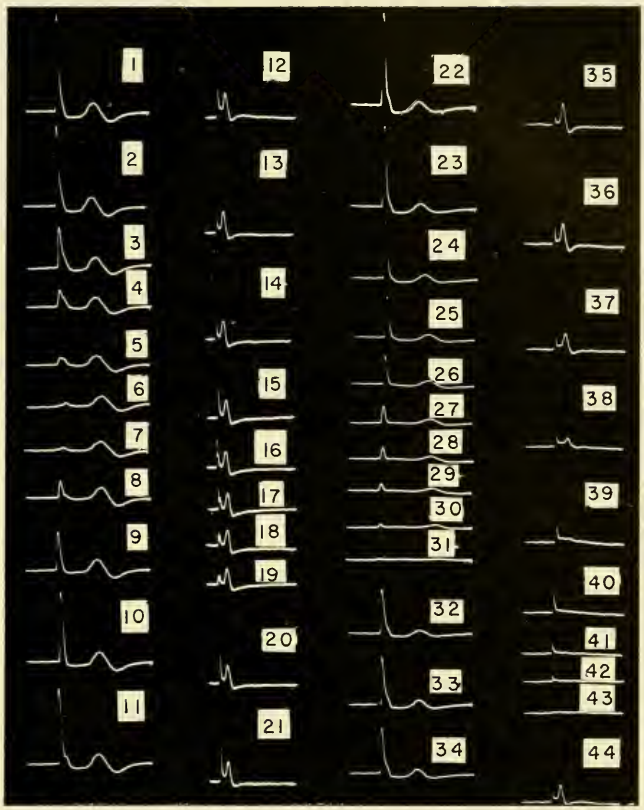


Fig. 7. A and B action potentials at low and normal temperatures. Records 1–21: blocking action of KCl at a concentration of 15.2 mM.

Records 1–11: temperature 1.1°C. Record 1 is control. Electrograms taken at 1′ (2), 3′ (3), 4′ (4), 5′ (5), 7′ (6), and 8′ (7) after test solution. Ringer wash occurred at 9′ and recovery records are shown for 11′ (8), 12′ (9), 13′ (10) and 18′ (11).

Records 12–21: temperature 22.7°C. Record 12 is the control. Electrograms 13–19 were obtained at 1′, 5′, 10′, 15′, 17′, 20′ and 25′ after adding test solution. Ringer wash occurred at 26′ and recovery records are shown for 27′ (20) and 28′ (21).

Records 22–44: responses to sodium. TMA was employed as osmotic substitute. Records 22–34: temperature 3.1°C. Record 22 is the control. Electrograms 23–31 were made at 3′, 7′, 9′, 10′, 11′, 12′, 13′, 14′, and 15′ after adding solution lacking sodium ions. At 33′ Ringer wash occurred and recovery is shown for 33′10″ (32), 34′ (33) and 36′ (34). Between 15′ and 33′ the responses to a solution with 11 mM NaCl were examined but these are not included in the figure.

Records 35–44: temperature 21.7°C. Record 35 is the control. Records 36–43 were taken at 4′, 9′, 13′, 15′, 19′, 22′, 24′ and 25′ after adding a solution lacking sodium ions. Ringer wash occurred at 35′ and recovery for 36′ (44) is shown. Between 25′ and 35′ the responses to a solution with 11 mM NaCl were examined but these are not included.

to excess potassium of the A and B fibers in the bullfrog sciatic nerve is maintained even at low temperatures. This statement is supported by the evidence in the electrograms of figure 7. Records 1 to 11 are the A and B waves obtained from a desheathed nerve at 1.1°C. Record 1 is the control obtained before adding the test solution to the nerve segment. A solution with KCl at 8.5 times the normal concentration was then added with the results shown in records 2–7. Over this period of time the A activity ceased almost entirely while the B potential was only slightly modified. Recovery took place rapidly and completely (records 8–11) following the addition of Ringer's solution to the nerve segment. The responses of the same nerve to the same test solution but at normal temperature (22.7°C) are arranged alongside in records 12–21. Only the time scale over which the action took place in the two experiments was different. The selective sensitivity of the A and B groups was not essentially altered by the low temperature. This similarity in the case of the responses to potassium is interesting because it is in sharp contrast to the behavior of the A and B groups to sodium.

RESPONSES TO SODIUM—A FIBERS

It is a commonly known fact that solutions with a deficiency of sodium ions produce a conduction block in nerve fibers (12, 17). The manner in which this low sodium block of the A fibers was influenced by cold is illustrated in figure 8. The four steps of this experiment were performed consecutively as numbered, using the normal temperature (22°C) for the initial step. For each of these steps a solution was added at zero time which had, in place of the NaCl of Ringer's fluid, an osmotically equivalent amount of TMA. The other constituents were present as in Ringer's solution. Conduction block was rapid in all four steps of this experiment and though there appears to be a slight slowing of the rate of block by low temperature, it is doubtful whether this represents a significant effect. In 13 experiments of this type (table 2) the time to 50% block at normal temperatures averaged 7.7 minutes with a range of 4.7–19.4 minutes. At the low temperature the average was 11.1 minutes and the range was 6.6–16.4 minutes. The difference was not at all striking and it is questionable whether any significance can be attached to the difference of the means. The more interesting and striking phenomenon was the manner in which recovery from the low sodium block was slowed or even completely inhibited by cold. These recovery effects were most conveniently studied, as indicated previously (5), not by adding Ringer's solution, but by means of a solution with a limiting concentration of NaCl. At normal temperatures a useful concentration for this purpose was found to be NaCl at 11 mM. With such a concentration of sodium ions it was previously found

(5) that activity could be restored in a majority of A fibers whereas solutions with slightly lower concentrations of NaCl caused either no recovery or only slight recovery. With such a limiting concentration of sodium ions it was observed that low temperature markedly retarded recovery of blocked A fibers. An example of almost complete inhibition is furnished by the data in curves 2 and 4 in figure 8. At normal temperature the solution with 11 mM NaCl yielded restoration of A activity to well over the

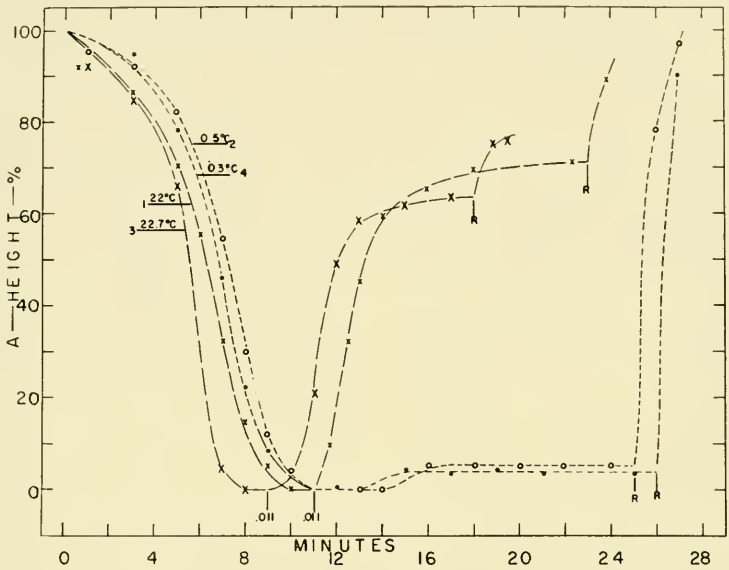


FIG. 8. Conduction block and recovery of A fibers at normal and at low temperatures. The four steps were carried out consecutively as numbered using the same nerve preparation. At zero time a solution without NaCl but with TMA as the substitute was added. At points indicated as .011 the test solution with NaCl at the limiting concentration was added. Ringer wash occurred at R. Temperatures are indicated by lines connected to each graph.

60% level (curves 1 and 3). At low temperature (curves 2 and 4) this test solution caused practically no restoration of action although Ringer's solution was perfectly effective. There is also evidence that the responses to sodium were also influenced by the nature of the osmotic substitute. As the data in table 2 suggest, recovery of the A fibers was less when TMA was employed than with choline. The retarding effect of cold on the recovery responses to sodium is interesting for it suggests the existence of a temperature-dependent system in the active membrane. Since the sodium conductance through the membrane depends on the membrane potential (14) it might be that cold exerts its effect by acting directly on this po-

tential. The precise relationship of the resting potential in bullfrog nerve fibers with temperature is not known. In the squid giant axon this relationship has been determined with some degree of certainty (13) and there appears to be no change in resting potential from 20°C down to 0°C.

Table 2. Low sodium block of a fibers and recovery with 11 mM NaCl

Normal temperatures						Low temperatures					
Nerve* No.	Normal Temp. °C.	Minutes to block to 25%	50%	75%	Recovery to 50%	Low Temp. °C.	Minutes to block to 25%	50%	75%	Recovery to 50%†	Osmotic Sub.
1	22.0a	4.3	6.2	7.2	2.2	0.5b	5.6	7.2	8.2		TMA
1	22.8c	4.0	5.7	6.5	3.4	0.3d	5.1	6.7	7.8		TMA
2	23.7b	4.0	6.2	7.5	3.1	1.8a	7.6	10.2	12.0		TMA
2	23.5d	3.2	4.7	5.7	3.4	3.2c	8.0	10.2	11.8		TMA
3	21.7b	13.2	19.4	22.0	2.6	3.1a	5.0	8.1	10.6		TMA
3	22.7d	10.2	12.7	14.2	2.8	6.8c	9.9	13.3	15.7		TMA
4	22.0b	8.0	10.9	12.9	4.2	3.2a	12.8	16.0	18.8	7.5	TMA
5	23.7a	3.6	5.0	6.2	3.7	4.5b	11.2	14.7	17.7	4.0	Choline
5	23.7c	4.9	7.2	8.8	2.7						Choline
6	23.7a	5.3	7.1	8.3	1.2	3.0b	7.1	9.0	10.6	4.2	Choline
6	23.7c	4.1	5.7	7.2	2.5						Choline
7	23.7a	4.6	6.3	7.8	1.4	2.0b	11.3	14.7	18.2	2.8	Choline
7	23.7c	5.7	7.3	8.7	2.0						Choline
8	23.7a	4.4	6.3	7.5	2.3	2.0b	9.3	11.7	13.4	3.0	Choline
8	23.7c	3.7	5.2	6.2	2.8						Choline
9	23.5b	3.9	5.2	6.5	0.8	2.0a	6.3	8.5	10.6	1.1	Choline
9						2.0c	10.5	13.0	15.8	1.0	Choline
10	23.7b	8.6	10.8	12.2	3.7	2.0a	10.7	14.3	17.3	2.0	Choline
11	23.7b	3.3	5.1	6.2	2.3	1.3a	4.4	6.6	7.9		Choline
11						2.0c	7.9	10.2	12.0	1.9	Choline
12	23.7b	4.3	7.5	9.2	1.0	2.0a	8.2	11.8	14.8		Choline
13	23.0b	5.2	7.4	9.0	1.7	2.0a	6.4	8.5	10.0	4.2	Choline
13	23.0d	7.0	9.6	11.2	1.7	2.0c	11.0	16.4	19.3	4.2	Choline
		5.5	7.7	9.1			8.3	11.1	13.2		

* The letters a, b, c, d, indicate the sequence in which the different stages were carried out.

† Where figure is not given recovery to 50% level did not occur.

Another possibility is that the gain in sodium ions which nerve fibers undergo at low temperature (25) could create an extra gradient against which the sodium ions must move in generating an action potential. It should be recalled, however, that the nerve employed for the experiment shown in figure 8 was, prior to the test with NaCl at 11 mM, exposed for 11 minutes to a solution entirely lacking in NaCl. Under these conditions

little uptake of sodium should be expected and possibly a loss of this cation actually occurred.

RESPONSES TO SODIUM-B FIBERS

Perhaps the most unexpected action of low temperature was in regard to the behavior of the B group of nerve fibers. As already indicated the B fibers at normal temperatures were more rapidly blocked by solutions lacking NaCl and required, for recovery, a higher concentration of sodium

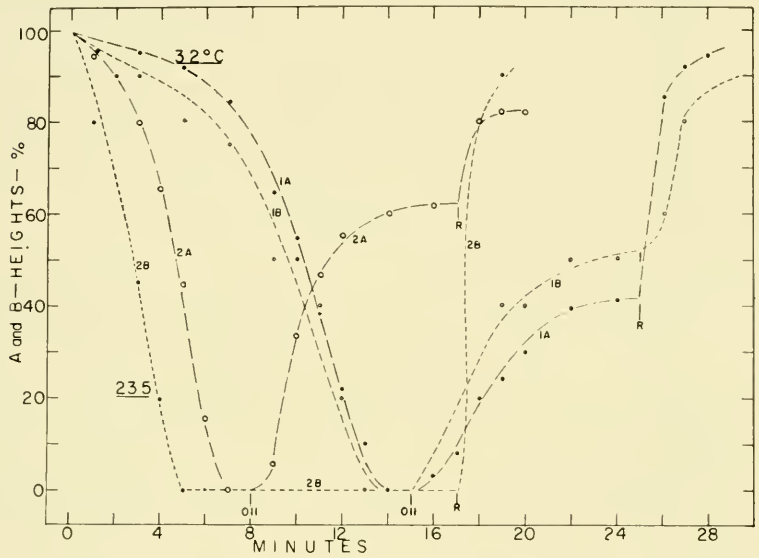


FIG. 9. Selective block and recovery of A and B fibers at 23.5° and 3.2°C. At zero time a solution without NaCl was added, using TMA as the osmotic substitute. At points indicated by .011 a solution with the limiting concentration of NaCl was applied. Ringer wash took place at R.

ions. Low temperature produced a significant modification in the reactions of the B fibers to sodium. One aspect of this modification is shown in records 22–44 of figure 7. These electrograms illustrate the point that at normal temperature the B fibers blocked significantly ahead of the A fibers (records 35–39). At the low temperature, on the other hand, the two groups blocked almost simultaneously (records 22–31). In other words cold led to a breakdown of the differential action of the low sodium solution. The graphs in figure 9 reveal another aspect of this modification. At 23.5°C (curves 2A, 2B) a typical low sodium cessation of action was observed with the B fibers (curve 2B) blocked well ahead of the A fibers (curve 2A). At the 8 minute point a solution with the limiting concentration of

NaCl (11mM) was added to the blocked nerve segment. This caused a typical selective response with the majority of the A fibers restored to activity while the B fibers remained in block until restored by the addition of Ringer's solution (at R). The same experiment performed with the nerve at 3.2°C (1A, 1B) gave entirely different results. In the first place the rate of block of the A and B groups was nearly the same. (In some experiments the A fibers actually blocked at a somewhat greater rate than did the B fibers.) In the second place, though the addition of the limiting concentration of NaCl resulted in a rate of recovery of A fibers slightly lower than the rate at normal temperature, it produced in the B fibers a considerable restoration of activity. As a matter of fact, the B group returned to action at low temperature at a rate faster than the A fibers. In other words, cold actually reversed the differential action of sodium in the restoration of these two groups of fibers. This behavior is singularly unlike the selective responses to excess potassium which, it will be recalled, were unaffected by reducing the temperature.

The action of cold in increasing the rate of low sodium block of the A fibers relative to the B group might have been the result of a leak and accumulation of potassium. Under these circumstances the A fibers, as already pointed out, being relatively more sensitive to potassium would tend to block faster, relative to the B fibers. It should be added, however, that this effect of cold was obtained under conditions where the treated nerve segment was immersed in fluid and underwent several washings during the addition of the test solution. The accumulation of potassium ions was therefore minimized. In any case this explanation cannot account for the recovery in the cold of many blocked B fibers following the addition of the limiting concentration of NaCl. It appears necessary to invoke some other action of low temperature in order to interpret the shift in differential action produced by cold. One obvious interpretation is to assume that the A and B fiber groups possess temperature-dependent systems in so far as the effectiveness of sodium ions is concerned. If the system for each of these two fiber groups is then assumed to have a temperature optimum with a fall-off on either side of this optimum as for a bell-shaped curve, then it is only necessary to make the additional assumption that the optima for the A and B groups are displaced along the temperature axis with the B optimum shifted toward lower temperatures. This scheme could account for the observed behavior to cold, for above the point of intersection of the two bell-shaped curves the A fibers would be relatively more sensitive to sodium ions whereas below the intersection the reverse would be true. This interpretation is interesting from one point of view, i.e. that it is so similar to the suggestion of Lundberg (18) who arrived at his conclusion from somewhat different types of experiments. He concluded, for

example, that the maximum membrane potential was at about 37°C for mammalian A fibers whereas it was at about 25°C for mammalian C fibers. Similar maxima for these fibers was found for the negative after-potential. The idea of temperature-dependent systems varying in this way is attractive, of course, because it calls to mind the behavior of enzymes and other proteins in relation to temperature.

RESPONSES TO SODIUM—ROLE OF THE OSMOTIC SUBSTITUTE

The low temperature experiments were instructive in demonstrating the importance of sodium concentration on the rate of conduction. This point is brought out for the case of the B fibers in the four experiments (columns A, B, C, D) of figure 10. These action potentials reveal, first of all, the slowing of conduction in the B fibers as low sodium block developed in the test nerve segment (records 1–7, 12–18, 23–27, 34–37). Following complete block in each case a solution was added which had the limiting concentration (11 mM) of NaCl. As already explained, such a solution was able, at the low temperature, to restore much of the potential. Such restoration is displayed in records 8–9, 19–21, 28–32 and 38–42. These records all bring out the extra conduction delay associated with the presence of a solution having a deficiency of sodium. Such a lowering in rate of conduction is expected under conditions of sodium deficiency because, for one thing, the rate of rise of the action potential is lower under such conditions (12). The influence of an increase in sodium concentration on conduction velocity is strikingly demonstrated in records 10, 22 and 33 which were obtained after the addition of Ringer's solution. This involved a change in NaCl concentration from 11 mM to 110 mM. This change, though it caused only little effect in height of B wave in some cases (records 10, 22), brought about a striking reduction in conduction time.

Another factor which appears to be involved in these responses to sodium is the nature of the so-called inert osmotic substitute, which is necessary in solutions deficient in NaCl. Both choline and TMA were utilized in separate experiments. Though the responses were qualitatively similar with these two organic ions, the recovery of B activity in the presence of the limiting concentration of NaCl was always greater with TMA. The comparison in behavior to these two ions is summarized in records 33–44 (fig. 10). Record 33 is the control with Ringer's solution in contact with the nerve segment. A solution containing choline chloride in place of the NaCl of Ringer's solution was then added to the nerve segment with the result shown in records 34–37. Following complete block of the B fibers a solution was then added which contained 11 mM of NaCl along with 99 mM choline chloride and the other constituents of Ringer's fluid. The B activity slowly returned (not presented in the figure) and its maximum

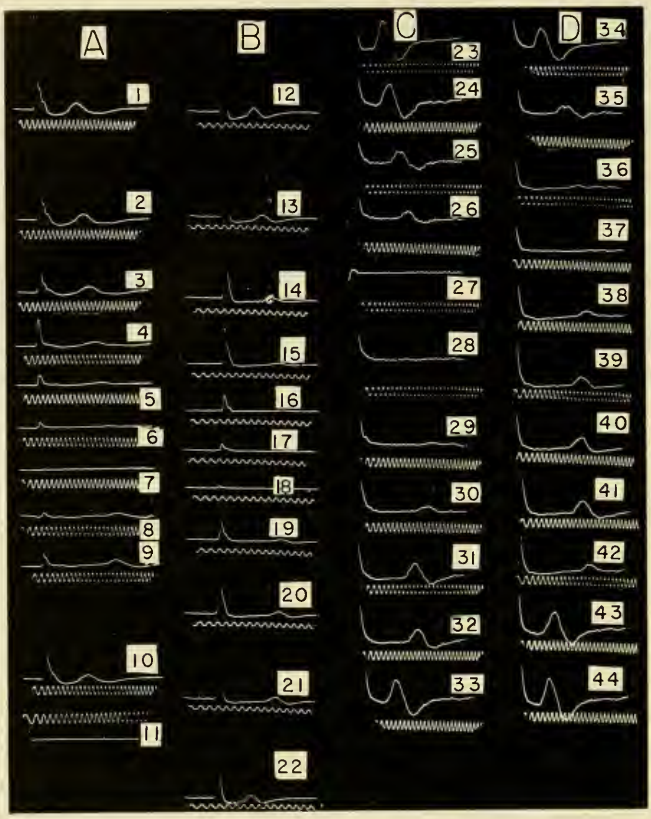


FIG. 10. Responses of B fibers at low temperatures. Four different experiments (A, B, C, D).

Column A: 4.2°C. Record 1 is the control. A solution with no NaCl but with TMA was applied and records 2–7 were taken at 3′, 7′, 8′, 9′, 10′ and 11′. At 12′ a solution with 11 mM NaCl (with TMA) was added. Responses 8 and 9 were taken at 13′ and 30′. Ringer wash occurred at 31′ and recovery for 32′ (record 10) is shown. All records have 60-cycle time line on lower beam. Record 11 shows this time line on upper beam.

Column B: 3.2°C. Record 12 is the control. A solution without NaCl (with TMA) was added and records 13–18 were obtained at 11′, 15′, 17′, 19′, 20′ and 21′. At 22′ a solution with 11 mM NaCl (with TMA) was applied. Records 19–21 are shown for 23′, 27′ and 35′. Ringer wash took place at 36′ and recovery for 38′ (22) is given.

Column C: 5.2°C. Record 23 is the control. The solution without NaCl (with TMA) was added and the responses (records 24–27) are given for 11′, 17′, 19′, and 25′. At 26′ a solution with 11 mM NaCl (with TMA) was applied. The responses (records 28–32) are listed for 27′, 29′, 32′, 35′ and 50′. Ringer wash occurred at 51′ and recovery is shown for 53′ (33).

Column D: 5.2°C. Continuation of *column C*. Record 33 is the control. A solution without NaCl but with choline chloride was added at 61′ and the responses (records 34–37) are given for 63′, 75′, 77′ and 78′. At 79′ a solution with 11 mM NaCl (with choline chloride) was applied. Recovery was allowed to proceed to its maximum and

restoration is indicated by record 38. Immediately after record 38 was taken the choline-NaCl solution was removed from the nerve segment and was replaced by another solution still with 11 mM NaCl but with TMA in place of the choline. Records 39–41 reveal the outcome of this exchange. The B activity was considerably increased and the conduction delay was decreased. One minute after taking record 41 the choline-NaCl was once again added to the nerve segment with again a reduction in rate of conduction and in height of B wave (record 42). The bromide ions of TMA were apparently not involved in the TMA effect since no difference in the responses to NaBr and NaCl has ever been observed (records 43 and 44).

It appears that the nature of the osmotic substitute was of some significance in these responses to sodium. This has already been noted (table 2) in connection with the A fibers in which, curiously enough, TMA and choline were found to behave in the reverse manner as in the case of the B fibers. Low temperature was not essential in establishing the point that the osmotic substitute modified the responses to sodium. In a previous study carried out at 23°C (5) it was noted, though no extended study was made of the phenomenon, that solutions containing 11 mM NaCl in the presence of sucrose were effective in restoring conduction in many B fibers. This did not occur with either choline or TMA. It is not a simple matter to attempt an interpretation of the activity of these so-called inert osmotic substitutes and to account for some of the effects which were observed. One thought is that certain organic ions may serve as substitutes, though varying in effectiveness, for sodium ions in the generation of the action potential. Lorente de Nó (17), in fact, has already postulated that certain quaternary ammonium ions may participate directly in the production of the nerve impulse and that sodium may actually possess an indirect function. Evidence gathered by the author (5) for the case of tetraethylammonium ions did not support this idea and certainly sucrose cannot be visualized as a substitute for either sodium or quaternary ammonium ions. Another interpretation is that these substances so modify the nerve membrane as to facilitate the action of sodium.

SUMMARY

It is likely that low temperature produced a multiplicity of effects on the complex system which constitutes the bullfrog nerve trunk. One of these—the leak and accumulation of a substance, probably potassium—

this is shown in record 38 taken at 106′. At 107′ a solution with 11 mM NaCl (but with TMA) was added. Records 39–41 were obtained at 108′, 109′ and 126′. At 127′ a solution with 11 mM NaCl (with choline chloride) was once more applied. The records were followed until no further change occurred and the final result is shown as record 42 taken at 145′. Recovery is shown first after addition of a solution with 110 mM NaBr (record 43) and finally after Ringer's solution (record 44).

was very likely the cause of the secondary decrease in action potential (fig. 3). Such a leak and accumulation possibly contributed a component to some of the other low temperature actions which were observed. The excess potassium block at low temperature (fig. 6) probably received a contribution from this source. For this investigation the experiments involved an attempt to minimize the accumulation of potassium by treating the nerve segment with several changes of test solution and by making each experimental run as short as possible. The nature of the evidence, already discussed in the body of this paper, leads to the thought that additional actions of cold, possibly direct effects on temperature sensitive systems, were also involved in some of the results. This statement has support from some data in the published literature. The initial increase in action potential with time (figs. 3 and 4) as the nerve cooled can be accounted for, at least in part, by the fact that the spike heights and spike areas of individual nerve fibers increase as the temperature is lowered. This fact is clearly brought out by the investigations of Schoepfle and Erlanger (22) and of Hodgkin and Katz (13). Both groups of investigators noted an augmentation in spike height of single fibers and a large increase in spike area as the result of lowering the temperature. The falling phase of the spike was found to be slowed more than the rising phase. Hodgkin and Katz reported Q_{10} values between 10 and 20°C to be 2.0 for the ascending phase and 3.2 for the falling period. Tasaki and Fujita (26) employed single fibers from the toad and obtained results which were in general agreement with the above data as far as the spike area was concerned. They reported an increase in duration of spike in passing from 20° to 5°C, the Q_{10} of the process being 3.5. With regard to spike height these workers observed, not an increase, but a small though continuous decline in cooling to 5°C. Another report on the effects of temperature on single spike activity is that of Hertz (11) who found for frog nerve fibers little change in spike height down to about 10°C. Below this value the height decreased slowly. A few investigators have employed nerve trunks for studies of the effects of temperature. Gasser's well-known study utilizing the sciatic nerve of the frog presented evidence of a notable decrease in compound spike below about 20°C (10). Lorente de Nó (15) employed the bullfrog sciatic nerve and noted an optimum temperature range for A-alpha fibers at 10°–15°C. There is, as indicated by this brief summary, some disagreement in the literature with regard to the details of the responses to temperature. This is not surprising since many factors probably determine the exact manner in which temperature will influence the height of the spike in a given preparation. Changes in resistance, ion shifts, length of exposure to low temperatures (figs. 3 and 4) and other factors are all involved. When comparing different types of nerve fibers or nerve fibers

from different animals additional factors come into play. Lundberg (18), for example, published records which demonstrated notable differences in temperature optima for mammalian A and C fibers. The phenomenon of temperature adaptation (2–4), unless recognized, could offer in different nerves from the same animal or the same nerve from different animals a source of troublesome variability. In spite of differences in the literature, the conclusion is clear that temperature-sensitive processes do exist in the sequence of events leading to spike formation.

Such processes are probably at the basis of the finding that cold selectively influences the sodium requirements for spike production in the A and B fibers of the bullfrog sciatic nerve preparation. The interpretation which was offered this phenomenon is that for both A and B groups of fibers there are processes with optimum temperatures—characteristically different for the two fiber groups—for the action of sodium in generating a spike. This idea is speculative and based only on the sodium responses of the A and B fibers at normal temperature and at low temperature. It should be possible in the future to test this idea by working out in an approximate manner the form and location of the temperature-sodium response curves for both A and B fibers. In the meantime there are the results of Lundberg (18) which suggest the same idea for mammalian A and C fibers. Lundberg recognized a number of objections to his procedures; namely, the use of different species of animals, the use of different nerves as a source of different fibers types leading, of necessity, to differential treatments of the various fiber types. These objections disappear in the present experiments since one was dealing here with different fibers in the same nerve and tested at the same time.

The notion of processes in nerve fibers with different characteristic temperature curves is of more than passing interest. Such systems are the sort of thing that one might expect to find in temperature receptors, for example. Such a motive is undoubtedly behind investigations such as that of Bernhard and Granit (1) who utilized peripheral nerve as a 'model temperature end organ'. The existence of characteristic temperature-sensitive systems in nerve cells is also of interest to the environmentalist who wishes to know if, and to what extent, environmental temperature changes can influence such systems and whether or not such effects are permanent in the organism. This aspect of temperatures analysis in biology has recently received an impetus from the studies of Chatfield and his group (2–4). They reported (2) that the tibial nerves from an hibernating mammal (the golden hamster) were able to conduct down to a temperature of 3.4°C whereas the comparable nerves from the non-hibernating albino rat ceased activity at 9.0°C. The curves relating action potential height with temperature were different for the two species. In another study (3)

it was reported that the tibial nerves of the albino rat stopped conducting at a temperature 4°–5°C higher than did the ventral caudal nerves from the same animal. No adaptation was noted in the case of the ventral caudal nerve. Perhaps the most interesting study of this series was the one (4) in which a gradient in temperature sensitivity was found for the superficial peroneal nerve of the Herring gull. Not only was the metatarsal portion of this nerve able to conduct at much lower temperatures than the tibial portion, but the metatarsal portion alone was able to adapt to an environmental change in temperature. These studies suggest that both adaptive and non-adaptive systems occur in nerve fibers and an analysis of these would appear to offer some hope of understanding the actions of temperature on nerve cells.

There are of course a number of observations made in the course of this investigation which are at present impossible to explain. Some of the effects may eventually find explanations in terms of some of the extraneous factors associated with whole nerve preparations. Others, however, may involve factors operating at the level of the excitable membrane. The cause of the differential interference with conduction in the A, B and C groups of fibers by drugs and ions continues to be a problem for which no solution is at hand. The nature of the selective action, in which some C fibers behave like the A group and other C fibers act with the B group, eliminates such factors as fiber size, surface relation, thickness of myelin, nodes of Ranvier and certain gross anatomical considerations. Then there are the curious observations showing that compounds like choline and TMA are, taken together, neither inert nor are they equivalent in their interactions with sodium. These might be considered as trivial types of responses if it were not for the fact that quaternary ammonium compounds are of physiological significance.

REFERENCES

1. Bernard, C. G. and R. Granit. Nerve as model temperature end organ. *J. Gen. Physiol.* 29: 257–265, 1946.
2. Chatfield, P. O., A. F. Battista, C. P. Lyman and J. P. Garcia. Effects of cooling on nerve conduction in a hibernator (golden hamster) and non-hibernator (albino rat). *Am. J. Physiol.* 155: 179–185, 1948.
3. Chatfield, P. O. and C. P. Lyman. Effects of temperature on the ventral caudal nerve of the rat. *Am. J. Physiol.* 177: 183–186, 1954.
4. Chatfield, P. O., C. P. Lyman and L. Irving. Physiological adaptation to cold of peripheral nerve in the leg of the Herring gull (*Larus argentatus*). *Am. J. Physiol.* 172: 639–644, 1953.
5. Crescitelli, F. Some features in responses of different nerve fiber types to a deficiency of sodium. *Am. J. Physiol.* 169: 1–10, 1952.
6. Crescitelli, F. Carbamate conduction block in frog nerve fibers. *Am. J. Physiol.* 155: 82–91, 1948.

7. Crescitelli, F. A temperature differentiation of the dual action of amyl carbamate on frog nerve. *J. Cell. & Comp. Physiol.* 35: 261–272, 1950.
8. Feng, T. P. and Y. M. Liu, The connective tissue sheath of the nerve as effective diffusion barrier. *J. Cell. & Comp. Physiol.* 34: 1–16, 1949.
9. Feng, T. P., C. H. Hsu and Y. M. Liu. The mechanism of the recovery of nerve asphyxiated in nitrogen when washed with O_2-free Ringer. *Chinese J. Physiol.* 17: 247–258, 1950.
10. Gasser, H. S. Nerve activity as modified by temperature changes. *Am. J. Physiol.* 97: 254–270, 1931.
11. Hertz, H. Action potential and diameter of isolated nerve fibres under various conditions. *Rasmus Navers Forlag,* Kobenhavn, 1–91, 1947.
12. Hodgkin, A. L. and B. Katz. The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol.* 108: 37–77, 1949.
13. Hodgkin, A. L. and B. Katz. The effect of temperature on the electrical activity of the giant axon of the squid. *J. Physiol.* 109: 240–249, 1949.
14. Hodgkin, A. L. and A. F. Huxley. The components of membrane conductance in the giant axon of *Loligo. J. Physiol.* 116: 473–496, 1952.
15. Lorente de Nó, R. A study of nerve physiology. *Studies from The Rockefeller Inst. for Med. Res.* 131–132: 1947.
16. Lorente de Nó, R. Effects of choline and acetylcholine chloride upon peripheral nerve fibers. *J. Cell. & Comp. Physiol.* 24: 85–97, 1944.
17. Lorente de Nó, R. On the effect of certain quaternary ammonium ions upon frog nerve. *J. Cell. & Comp. Physiol.* 33: Supp., July, 1949.
18. Lundberg, A. Potassium and the differential thermosensitivity of membrane potential, spike and negative afterpotential in mammalian A and C fibres. *Acta physiol. scandinav.* 15 *Supp.* 50, 1948, 1–67.
19. Lundberg, A. On the ability of some cations to inhibit the potassium depolarization of frog nerve fibers. *Acta physiol scandinav.* 22: 365–375, 1951.
20. Lundberg, A. On the effect of temperature on the depolarization of frog nerve fibers. *Acta physiol. scandinav.* 22: 348–364, 1951.
21. Lundberg, A. On the mechanism of differential sensitivity of mammalian nerve fibers to depolarizing agents. *Acta physiol. scandinav.* 26: 156–173, 1952.
22. Schoepfle, G. M. and J. Erlanger. The action of temperature on the excitability, spike height and configuration and the refractory period observed in the responses of single medullated nerve fibers. *Am. J. Physiol.* 134: 694–704, 1941.
23. Shanes, A. M. Potassium movement in relation to nerve activity. *J. Gen. Physiol.* 34: 795–807, 1951.
24. Shanes, A. M. Effect of temperature on potassium liberation during nerve activity. *Am. J. Physiol.* 177: 377–382, 1954.
25. Shaw, F. H. and S. E. Simon. The nature of the sodium and potassium balance in nerve and muscle cells. *Australian J. Exper. Biol. & M. Sc.* 33: 153–177, 1955.
26. Tasaki, I. and M. Fujita. Action currents of single nerve fibers as modified by temperature changes. *J. Neurophysiol.* 11: 311–315, 1948.

INFLUENCE OF CHANGES IN TEMPERATURE AND PRESSURE ON THE NERVE FIBER[1]

Ichiji Tasaki and Constantine S. Spyropoulos, *Laboratory of Neurophysiology, National Institutes of Health, Bethesda, Maryland*

If nervous activity depends on biochemical reactions, the rates of various phases of this activity should, in general, be modified by changes in temperature and pressure. Although the demonstration of a marked temperature or pressure dependence of a biological process may not prove that this process is carried out by chemical reactions, it does serve to distinguish it from other processes having different types of temperature or pressure dependence.

The effects of temperature changes upon various phases of nervous activity have been investigated by a great number of workers. In 1908, Keith Lucas (1) showed that conduction velocity in the frog motor nerve decreased with a fall in temperature; the Q_{10} he obtained was approximately 1.8. This result was confirmed by many recent investigators. In 1912, Adrian (2) found that the duration of both the absolute refractory period and of the electric response of the frog nerve varies with temperature with a Q_{10} of approximately 3. A little later, Gasser (3) made a series of classical observations on the effects of temperature upon various phases of the action potential of the frog nerve. More recently the effects of temperature changes upon the frog nerve fiber have been investigated by Bremer and Titeca (4), Schoepfle and Erlanger (5), Lorente de No (6), Lundberg (7), Tasaki (8–10), and many others. The temperature effects on the isolated invertebrate axon have been reported by Cardot and Arvanitaki (11), Hodgkin and Katz (12) and others.

Investigations on the effects of pressure changes upon the nerve are very scarce. Grundfest and Cattell (13) in this country and Ebbecke and Schaefer (14) in Germany made some pioneer observations in this field. The investigation along this line was, however, completely interrupted for almost two decades.

This article is a review of the experimental studies on the effects of temperature and pressure changes upon the isolated myelinated nerve fiber of the frog and toad and upon the isolated giant axon of the squid.

---

[1] Part of the work presented in this article was carried out at the Marine Biological Laboratory, Woods Hole, Massachusetts.

The discussion will be limited to the experiments on isolated single fiber preparations since the results obtained with whole nerve trunks are often very difficult to interpret unless the behavior of the constitutent fibers is clearly understood. The discrepancy between Gasser's results (3) and the results of Schoepfle and Erlanger (5) on the temperature dependence of the amplitude of the action potential is an example of this kind of difficulty.

In this article attempts are made to compare the results obtained with the squid axon with those obtained with the amphibian nerve fiber. In general, experiments on the squid axon are easier and the results obtained are more direct than the corresponding experiments and results on the frog myelinated nerve fiber. Since, however, the membrane constants, cable properties and the potential-impedance relationship of the vertebrate nerve fiber are very different from those of the squid axon, it is not always safe to infer the behavior of the frog nerve fiber from experiments on the squid axon. An effort has been made, therefore, to obtain direct experimental data from both of these types of nerve fibers. A portion of our unpublished work described in this article will be discussed more fully in subsequent papers.

METHODS USED IN EXPERIMENTS ON MYELINATED NERVE FIBERS

Isolation of Single Nerve Fibers. The nerve innervating the semitendinosus or the sartorius muscle of the toad (*Bufo marinus*) or the bullfrog (*Rana catesbiana*) was used. A segment 1–5 mm long of the nerve was desheathed and all except one of the fibers in the desheathed region were cut. The fiber selected was, as a rule, motor, and 10–15 μ in diameter. The dissecting instruments were a pair of needles mounted on wooden holders. The entire operation was carried out under dark field illumination (for greater detail, cf. 15).

Measurement of Action Current. Three different methods were employed for measuring the action current of the myelinated nerve fiber. In the *bridge-insulator method* (fig. 1A), the isolated single fiber preparation was mounted on a platform (bridge-insulator) made of two glass plates fixed side by side on a lucite stage. The gap between the two plates measured 0.1–0.2 mm. The segment of the nerve on one side of the dissected region was placed on one glass plate and the portion on the other side was mounted on the other plate. In this manner a thickly myelinated internode of the isolated fiber 'bridged' the gap between the two plates. On either side of the gap the isolated fiber preparation was bathed in a pool of Ringer's solution. An electrode of the Ag-AgCl-Ringer (agar) type was immersed in each pool of Ringer. One of the electrodes was grounded either directly or through a 1000-ohm resistor and the other was led to the input of a preamplifier. A 300-kilohm resistor was connected across the terminals

of the preamplifier. The potential drop across this resistor gave a measure of the action current.

In the *interface method* for recording the action current (fig. 1B), the single fiber preparation was suspended in a vessel containing Ringer's solution below a layer of mineral oil. The portion of the nerve on one side of the operated region was attached to a non-polarizable grid electrode located in the layer of mineral oil. The portion of the nerve on the other

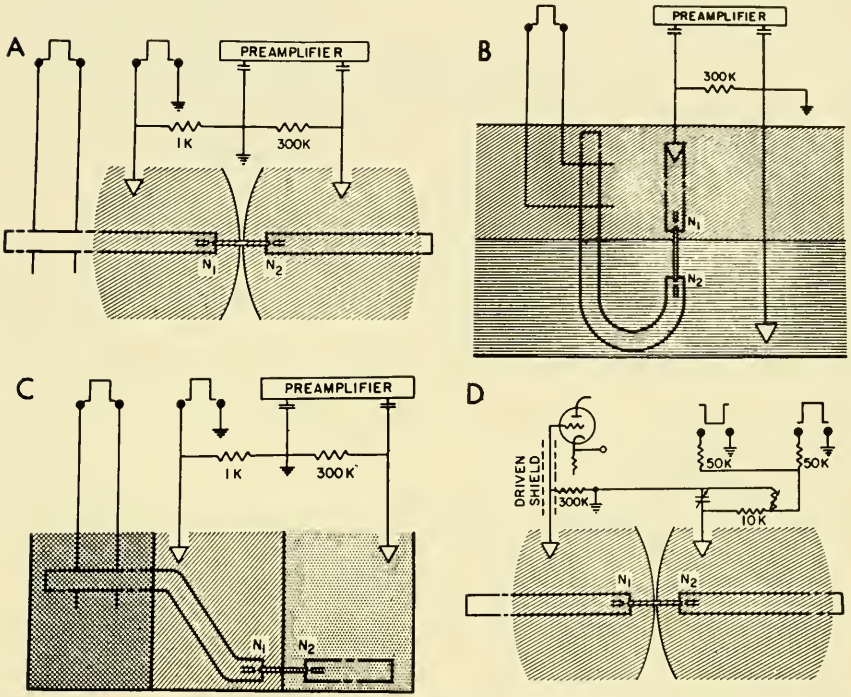


FIG. 1. Four different methods used for stimulating a single myelinated nerve fiber and for recording its action currents.

side of the operated region was immersed in the Ringer. The Ringer fluid was grounded by another non-polarizable electrode. The preparation was mounted in such a way that the oil-Ringer interface crossed one of the isolated internodal segments.

In the third method, the *three-compartment method*, of recording action currents of a single fiber the preparation was mounted in a chamber composed of three relatively large compartments (fig. 1C). One lateral compartment was filled with mineral oil, the middle compartment with normal Ringer and the other lateral compartment with either normal

Ringer or cocaine-Ringer. The partitions between the compartments measured about 0.5 mm in thickness. After the single fiber preparation was passed through small holes in the partitions, these holes were sealed with vaseline.

In all three of these methods of recording the action current, the input resistance R (300 K in the figure) was situated in such a position that the longitudinal current, I, flowing through the internode between N_1 and N_2 produced an IR drop across this resistance. The voltage at the input of the preamplifier varied directly with the value of R in a wide range of R, indicating that the action current I is independent of the input resistance R. Since, however, the response time of the input stage of the preamplifier increased with increasing R (due to the capacity of the recording electrode and of the preamplifier input to ground), it was not possible, under ordinary experimental conditions, to increase R above 1 megohm without losing high-frequency components in the response. When the excitability of the node N_2 was eliminated by the use of cocaine, action currents that roughly parallel the membrane potential at N_1 were obtained.

Measurement of Action Potential. The isolated fiber in the dissected region of the nerve was suspended across the gap between a glass plate and a small electrode of the Ag-AgCl type (fig. 2A). The air gap measured 0.5–1 mm. The portion of nerve on the glass plate was immersed in normal Ringer. The electrode that was immersed in the pool of Ringer on the glass plate was grounded (through 500 ohms).

The cathode-follower used for this purpose had an effective input capacity of about 1 $\mu\mu$f or less. The details of a high input-impedance preamplifier especially designed for this purpose in this laboratory will be presented elsewhere (16). The grid electrode was completely enclosed in a metal shield driven by the output of the preamplifier which had a gain of unity. After eliminating the excitability of the node (N_2) on the grid electrode with an isotonic KCl solution, both the resting and the action potential of the fiber could be measured directly without any appreciable distortion or reduction. The resistance of the preparation (measured by using a square voltage pulse across the 500-ohm resistor and the 22-megohm resistor at the input) was between 30 and 60 megohm.

The action potential of the individual nerve fiber can also be recorded by the use of a submicroscopic microelectrode. But, at present, the results obtained with submicroscopic electrodes are less reliable than those obtained by the method described above.

Measurement of Membrane Resistance and Capacity. The principle of the method used for measuring the resistance and capacity of the myelin sheath and the nodal membrane is illustrated in figure 2B. The single fiber preparation was mounted on a platform consisting of three pools of

Ringer divided by two narrow air gaps. The width of the middle pool was 1 mm and that of the air gaps approximately 0.1 mm. When the property of the nodal membrane was to be examined, an exposed node of the preparation was introduced into the middle pool. The section of fiber on this pool was immersed in choline (sodium-free) Ringer. For measurements

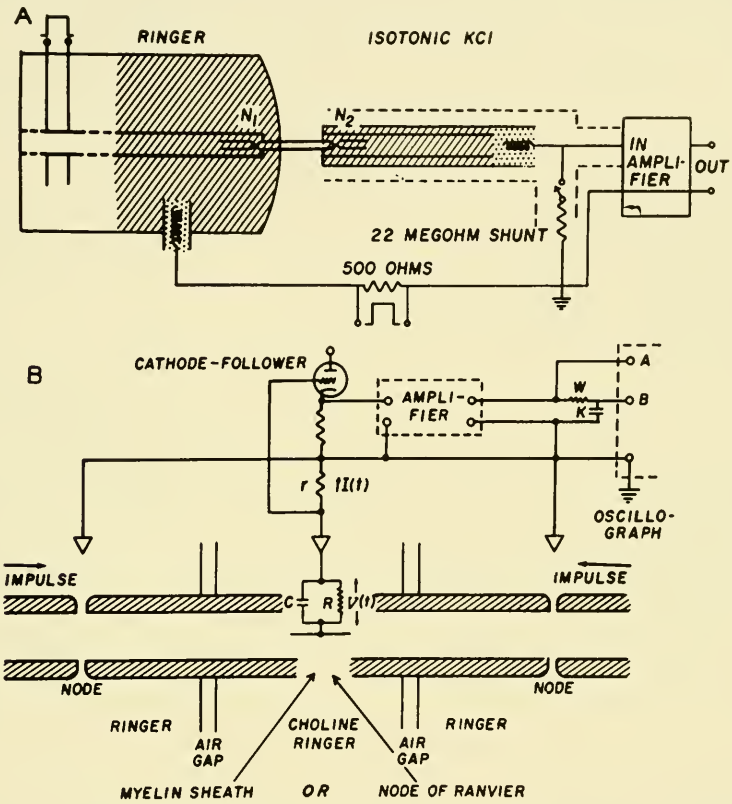


FIG. 2. *A:* diagram illustrating the method of recording the action potential of a single myelinated nerve fiber. *B:* diagram showing the method of measuring the resistance and the capacity of the myelin sheath and of the nodal membrane.

of the physical constants of the myelin sheath, only the myelinated portion of the preparation was immersed in the middle pool. The two lateral pools were filled with normal Ringer and were directly grounded with large electrodes. The small middle pool was grounded through resistance r in the figure.

When two impulses arrived simultaneously at the nodes located in the lateral pools (see figure), the myelin sheath in the middle pool was trav-

ersed first by an outward current and then by a weak inward current. This membrane current was considered to consist of a capacitative component (flowing through C in the figure) and an ohmic component (flowing through R). The nodal action potential (a rounded triangle with a peak value of 100–110 mv) was uniform and the variation among different fibers was found to be small (10). It was possible therefore to determine the values of the capacity and the resistance by separating the membrane current, I(t) in the figure, into its capacitative and ohmic components (for more details of the principle of this method, cf. 17). The principle of the method for determining the capacity and the resistance of the nodal membrane was similar to that for the myelin sheath with the added complication that the membrane potential at the inexcitable node in the middle pool was appreciably smaller than the action potential of a normal node. In the frog myelinated fiber, the change in the membrane resistance with increasing membrane potential was far smaller than the change shown by Cole (18) in the squid axon.

Measurement of Membrane Impedance During Activity. The change in the membrane impedence during activity was measured by an A.C. Wheatstone bridge. The single fiber preparation was mounted on a bridge-insulator as in figure 1A. One arm of the A.C. bridge consisted of the preparation and the electrodes in the pools. The distal node, N_2, was made inexcitable with a narcotic. The change in the membrane impedance associated with the action current of the node N_1 was detected as a change in balance of the bridge. The frequency of the bridge A.C. was between 2 and 6 kc. This type of impedance measurement was successful only in the range of temperatures below about 10°C, that is, only when the period of the bridge A.C. was far shorter than the duration of the nodal activity.

Measurement of Threshold. The threshold voltage (rheobase) for the fiber was measured by the use of long rectangular voltage pulses applied across the 1000-ohm resistor in figure 1A. With this arrangement the resistance of the internodal segment between N_1 and N_2 was connected in series with the source of the applied stimulus. Under ordinary experimental conditions, a change in the threshold voltage was a measure of a change in the threshold intensity of current through the nodal membrane.

Thresholds for short voltage pulses were determined by using the same arrangement (fig. 1A). Under the conditions of this experiment, the rise in threshold with decreasing stimulus duration was determined primarily by the capacity of the myelin sheath and the nodal membrane near the stimulating air gap. The classical chronaxie is a measure of the time required to charge the nodal membrane up to a certain level which is practically independent of the stimulus duration.

Thresholds for exponentially or linearly rising voltage pulses were measured by the arrangement of figure 1D. The variable resistance in the

stimulating circuit was smaller than 1000 ohms. The time constant of voltage rise was, therefore, determined by the product of the capacity of the variable condenser and the 10-kilohm resistor in the figure. The final value of the exponentially rising voltage was given by the voltage divider consisting of the fixed 50-kilohm resistor and the variable resistor. A linearly rising stimulating voltage was produced by adjusting the duration of the square voltage pulse to a value far shorter than the time constant of the exponentially rising potential. By starting the second pulse of the opposite polarity at various intervals after the onset of the first pulse, triangular voltage pulses were produced.

METHODS USED IN MEASUREMENTS ON SQUID AXONS

Measurement of Resting and Action Potential. The well-known method of introducing a saline-filled glass pipette longitudinally into an isolated axon (19, 20) was employed. Both the axon and the intra-cellular glass pipette were fixed to a glass slide so that the preparation could be transferred from one vessel of sea water to another. Stimulating shocks were applied to the axon near its end through a pair of metal (usually steel) wire electrodes.

Measurement of Membrane Resistance and Capacity. A pair of intra-cellular metal wire electrodes were used, one wire for passing a current pulse through the axonal membrane and the other for measuring the change in membrane potential caused by the current. The current electrode was usually made from a fine enameled silver wire. The enamel coat at the tip of the current electrode was scraped off for a length of about 10 mm. This electrode was inserted longitudinally into an isolated axon. The axon was about 40 mm in length. The exposed portion (about 2 mm in length) of the potential electrode was located in the middle of the scraped portion of the current electrode. A precaution was taken to avoid direct metallic contact between the current and potential electrodes.

Measurements were made in three ways. In one case, a strong short pulse of inward current was applied to the membrane to charge the capacity and the exponential decay of the membrane potential was followed; the initial rise in potential was a measure of the capacity and the rate of the exponential decay was a measure of the time constant of the membrane. The second method of measuring the membrane resistance and capacity was to pass a weak constant current through the membrane and to follow the change in membrane potential; the initial rate of potential change was a measure of the membrane capacity and the final level of the potential, a measure of the membrane resistance. The third method employed was the 'method of voltage clamp' used extensively by Hodgkin and Huxley (21). The membrane potential was suddenly clamped at a new level, and the intensity of the membrane current necessary to main-

tain this new level was recorded. By changing the level of the new membrane potential step by step, a measure of the membrane resistance was obtained.

Measurement of Membrane Impedance During Activity. An A.C. impedance bridge was constructed, one arm of which consisted of the squid axon membrane and a pair of electrodes (one inside and the other outside the axon). The internal electrode was a 100 μ silver wire with its enamel coating removed for a length of about 10 mm. The ratio arms of the bridge consisted of a pair of fixed resistors, one 10 ohms and the other either 100 or 300 ohms. The bridge was balanced by varying the resistance (0–300 kilohms) and the parallel capacity (0–200 $\mu\mu$f) in the third arm of the bridge. The frequency of the bridge A.C. was 20 (sometimes 10) kc per sec.

METHOD USED FOR APPLICATION OF PRESSURE TO NERVE FIBER

Hydrostatic pressures up to 16,000 psi were developed by the use of a hand-operated hydraulic pump (fig. 3). The reaction vessel (pressure

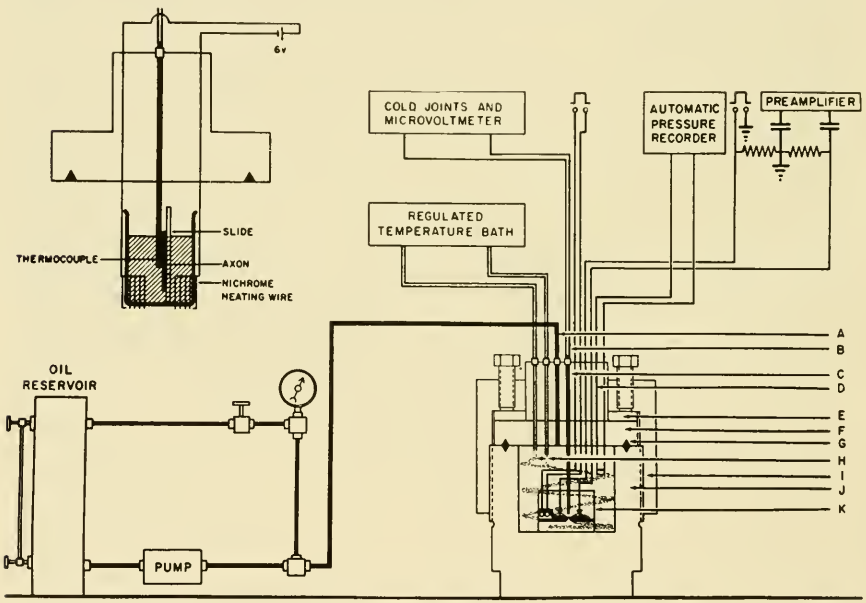


Fig. 3. Experimental set-up used for applying high hydrostatic pressures to the nerve fiber. *A:* superpressure tubing connecting pump to interior of reaction vessel; *B:* self-sealing electrical connectors between inside and outside of reaction vessel; *E:* steel thrust ring; *F:* inner pressure head; *G:* 'delta' type steel gasket; *H:* coils made of superpressure tubing for circulating hot or cold fluid through the interior of the reaction vessel; *I:* outside cap screwed on to body of vessel (The cap was equipped with 16 thrust bolts); *J:* body of reaction vessel; *K:* bridge-insulator.

bomb) was of the 'outside-cap compression closure' type. The inside diameter of the vessel measured $5\frac{1}{16}$ in. and the depth of the vessel 10 in. Six self-sealing electric connectors were installed in the inner pressure head. The pump, reaction vessel and the connecting ¼-in. superpressure tubing were filled with mineral oil. The glass vessel containing a single fiber preparation in a volume of Ringer (or sea water in case of a squid axon) was immersed in the mineral oil in the pressure bomb.

The coefficient of compressibility and the specific heat of the mineral oil are such that with an increase in pressure of 15,000 psi the temperature of the oil may increase by as much as 10 degrees. The rise in temperature of sea water or Ringer was (for the same increase in pressure) less than $\frac{1}{7}$th the rise of mineral oil. In order to minimize the effect of a change in temperature due to compression, it was necessary to avoid immersing the nerve fiber directly in mineral oil. Equilibration at the desired temperature was achieved by the flow of water, from a regulated temperature bath, through a coil of super-pressure tubing surrounding the nerve preparation.

EFFECTS OF TEMPERATURE CHANGES ON NERVE FIBER

Resting Potential. The effect of temperature changes upon the resting membrane potential of the isolated myelinated nerve fiber has not yet been investigated. On the squid giant axon, Hodgkin and Katz (12) have shown that the resting potential is practically independent of the temperature in the range between 0 and 20°C. The resting potential of the skeletal and cardiac muscle has also been found to be only slightly affected by temperature changes (22–24).

Membrane Capacity and Resistance. The capacity of both the myelin sheath and the nodal membrane has been found to be practically independent of temperature in the range between 14° and 25°C (17). Similarly the capacity of the squid axon membrane did not show any appreciable temperature dependence between 3° and 23°C.

The membrane resistance, on the contrary, showed an appreciable temperature dependence in the frog myelinated fiber (17). The resistance of both the myelin sheath and the nodal membrane (in choline) increased by 50 to 80% when the temperature was lowered from 25° to 15°C. An equally strong temperature dependence of the membrane resistance has been observed by Tamasige (25) in the muscle fiber of the frog. Del Castillo and Machne (26) however using a different method reported a slightly lower Q_{10} (1.35) for the muscle fiber membrane. Coraboeuf and Weidmann (23) found a Q_{10} of 1.49 for the membrane resistance of Purkinje fibers. In the squid giant axon the membrane resistance at rest was not appreciably affected by temperature changes. In the range between 5° and 25°C, the increase in the resistance for a fall of 10° was about 9–25%. Since the squid

axon membrane resistance is not independent of current (18), there is some arbitrariness in this measurement.

This relatively significant difference in the temperature dependence of the resting membrane resistance between the frog nerve fiber and the squid axon could be ascribed to the existence of many cut branches in the latter fiber. It is our impression that there were in the area of 1 cm^2 of the giant axon membrane (i.e. in the region of 6–7 cm length) usually 10–30 cut branches. The diameter of these branches was often larger than 50 μ. If the regions of the membrane where the branches were cut were to be considered as holes devoid of any highly resistive cap, then the sum of the electric conductance of these holes cannot be far smaller than the conductance through the intact portion of the membrane. If this be the case we have studied the low temperature dependence of the shunting holes rather than that of the 'membrane' proper.

Resistance of the Axoplasm. There is at present no accurate measurement of the temperature dependence of the axoplasmic resistance on the frog myelinated nerve fiber. Hodler, Stämpfli and Tasaki (27) have shown that sudden cooling of a part of an internodal segment caused a decrease in the size of the action current recorded across the same internode. From these experiments it seems very likely that the axoplasmic resistance increased with a fall in temperature. A strong temperature dependence (Q_{10} of about 2) of the resistance of the sarcoplasm of the frog muscle fiber was reported by Tamashige (25). Coraboeuf and Weidmann (23) reported a Q_{10} of 1.48 for the myoplasmic resistance of the Purkinje fibers. In the squid giant axon, a recent measurement by Schmitt (28) showed that the axoplasmic resistance increases by about 30% for a fall in temperature of 10° (between 5° and 25°C).

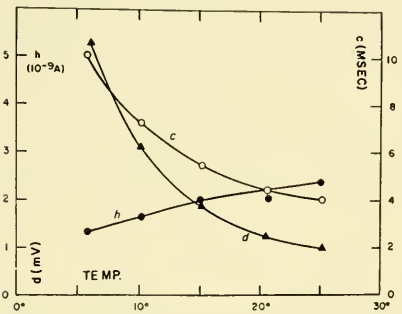
Action Potential. In the range of temperature between 5° and 25°C, the spike amplitude of the action potential of the myelinated nerve fiber of the toad was found to be 100–110 mv (10, 16). In the squid giant axon, Hodgkin and Katz (12) showed that the spike amplitude increases very slightly (5–10%) with a drop in temperature from 20° to 2°C. Similarly in the cardiac muscle the spike amplitude of the action potential was found to be independent of temperature (23, 24).

The spike duration of the action potential, on the contrary, was found to increase significantly with a fall in temperature. In the toad motor nerve fiber (8), the value of the Q_{10} was about 3.5 (see *curve d* in fig. 4). In the squid giant axon, a Q_{10} of 3–3.5 has been reported for the falling phase of the spike potential (12). The rising phase of the action potential is known to show a smaller temperature dependence than the falling phase; in the squid axon, it has been reported that the Q_{10} is close to 2 (12). In the frog nerve fiber, however, the shape of the rising phase was determined

primarily by the amount of capacitative distortion by the myelin sheath between the node under investigation and the grid electrode and did not reflect the rapidity of the process at the node. It is not possible at present to determine the temperature effect upon the rising phase of the nodal action potential.

The end of the absolute refractory period of a node was found to coincide fairly well with the end of the nodal action potential (15, 2). Since this was true at all the temperatures examined, it is clear that the value of Q_{10} for the duration of the absolute refractory period is close to 3.5.

It should be pointed out in this connection that the classical refractory period, i.e. the least interval between two shocks necessary to produce two



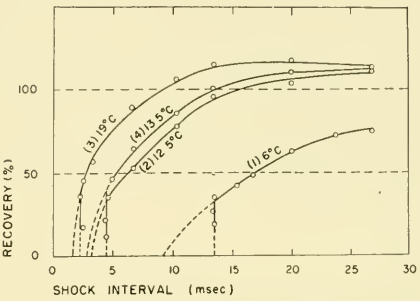


FIG. 4. *Left:* Effects of temperature upon the duration of the action current (d), the amplitude of the action current (h) and the shock-response interval (c) of a motor nerve fiber of the toad. From *J. Neurophysiol.*, 11: 1948.

FIG. 5. *Right:* Recovery of excitability of a single nerve fiber of the toad measured at four different temperatures. The points at which the dotted curves cross the abscissa indicate the absolute refractory period of the node. From *Biochim. et biophys. acta* 3: 1949.

propagated responses, is slightly longer than the true refractory period of the node (15). In figure 5 the effect of temperature changes upon the absolute and relative refractoriness of the toad nerve fiber is illustrated.

The end of the squid action potential is obscured by the strong 'undershoot' which follows the main spike. Nevertheless, the Q_{10} for the duration of the absolute refractoriness appeared to be similar to that for the spike duration.

Action Current. Since the action current is a flow of electricity associated with the production of the action potential, its time course is determined by the shape of the membrane action potential and the distribution of resistances and capacities inside and outside the fiber. In the frog motor nerve fiber, the rate of potential rise at an active node is very high (approximately 1500 volts per sec.) and the time constant of the myelin

sheath (0.4–0.5 msec. at room temperature) is far longer than the rising phase of the nodal action potential (17). The initial part of the action current of the fiber, therefore, consists of a purely capacitative flow of current through the myelin sheath between the node in action and the recording partition.

Previously, one of us (8) reported that the spike amplitude of the action current recorded by the technique of figure 1A decreased with a fall in temperature at a rate of about 30% for a change of 10 degrees (see *curve h* in fig. 4). Recently, Maruhashi (personal communication) found that this temperature dependence of the amplitude of the action current became negligibly small when the distance between the recording partition and the active node was reduced to about 0.1 mm. Since the resistance of the axis-cylinder varied and the capacity of the myelin sheath did not vary with temperature changes, it is not surprising that the temperature coefficient of the amplitude of the action current was dependent upon the length of the axis cylinder between the active node and the recording partition.

In the squid giant axon, the current I(t) that flows through the axoplasm when an impulse travels along the axon at a uniform velocity v is given simply by

$$I(t) = \frac{1}{vR}\frac{dV(t)}{dt}$$

where V(t) represents the time course of the action potential and R the resistance per unit length of the axoplasm. The temperature dependence of V(t) and R have been discussed above. As to the temperature effect on the impulse velocity v, we could not find any accurate information in the literature. It is clear, however, that the temperature effects on the remaining two terms in the above expression, i.e. R and dV(t)/dt, would tend to reduce the current I(t) with falling temperature.

Membrane Resistance During Activity. In the range of temperature between 3° and 10°C, the time course of the impedance-loss during activity of a nodal membrane was roughly parallel to that of the nodal action potential or action current (30, 31). The duration of the period of decreased membrane impedance has, therefore, the same temperature dependence as the spike duration. It is not known whether the maximum impedance-loss during activity of a node varies with temperature or not.

In the squid giant axon, the maximum impedance-loss during activity was found to decrease with a fall in temperature. The duration of decreased membrane impedance was found to have a temperature coefficient of the same order of magnitude as that of the duration of the falling phase of the action potential. No attempt was made to estimate the absolute value of the membrane resistance at the peak of activity by the A.C. method. By the method of voltage clamp, it was found that at 22°C the

membrane resistance at the peak of activity was about 10 ohm·cm^2. At about 10°C, the membrane resistance at the peak of activity was approximately 60% higher than the value at room temperature.

Thresholds. The word 'threshold' does not mean much unless one specifies *a*) the manner in which the stimulus was delivered to the nerve fiber and also *b*) the time course of the stimulating current or voltage. With the experimental set-up of figure 1A and using rectangular voltage pulses across the two pools as stimuli, it was found that with a fall in temperature the rheobasic voltage (threshold for pulses of 10 msec. duration) remained almost unchanged (or sometimes was slightly decreased) while the threshold for short pulses increased considerably. With extremely brief shocks (for which the constant quantity law holds) the threshold doubled for a decrease in temperature of 13 degrees.

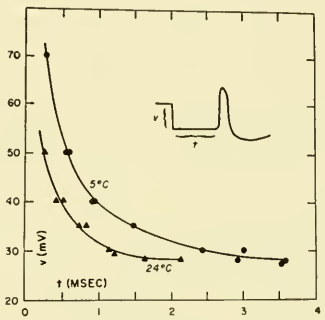


FIG. 6. Effect of temperature upon the strength-latency relation of the node of Ranvier. V indicates strength of stimulus in mv, and t, the time from the beginning of the stimulus to the appearance of the response. From *J. Neurophysiol.* 11: 1948.

The temperature dependence of the threshold membrane potential at the node (29) has not been accurately measured. But, since both the axoplasmic resistance and the resistance of the nodal membrane increased as temperature was lowered, the temperature independence of the rheobasic voltage mentioned above indicates that the threshold membrane potential did not vary appreciably with temperature. When only a part of an internode of a fiber was cooled, the rheobasic voltage measured at this cooled internode was appreciably increased (27).

The strength-duration relation of a fiber (i.e. the relation between the threshold strength and the stimulus duration) resembled very closely the strength-latency relation obtained under the same experimental conditions. In figure 6 is shown the relationship between the latency of action current, t, and the voltage, v, of a long rectangular pulse measured by the method of figure 1A at two different temperatures (8). It is clear that the latency prolonged markedly as temperature was lowered. This effect was completely reversible unless the nerve fiber was maintained at extreme temperatures for a long period of time. Our present interpretation of this temperature effect upon the latency is as follows.

When a rectangular voltage pulse is applied across the air gap in the experimental arrangement of figure 1A, the voltage across the nodal membrane is expected to rise first slowly, then quickly and finally to approach a steady level (32). The time required for this sigmoid ascent of the membrane potential is determined primarily by the cable property of the myelinated nerve fiber. The capacities of the nerve fiber do not change with temperature, but the resistances increase as temperature drops. For this reason, the time required for the spread of potential increases with a fall in temperature. Even at about 5°C, the process of excitation at the node appears to progress in a period of time much shorter than the time required under these experimental conditions to raise the membrane potential to the threshold value (31, 28). A recent observation on the action potential of a single node (29) is in good agreement with the view that the action potential starts approximately at the moment when the membrane potential reaches the critical, threshold level. It is reasonable, therefore, to attribute the change in both the strength-latency and strength-duration relations with temperature mainly to the change in the cable constants of the resting nerve fiber.

It is well known that a nerve fiber fails to respond if the stimulating voltage is increased at a rate smaller than a certain minimal gradient. This minimal gradient, which was determined with the arrangement of figure 1D, decreased with a fall in temperature (9). A strange fact about the temperature dependence of the minimal gradient is that, unlike the spike duration and thresholds for brief shocks, it shows a definite hysteresis with changing temperatures (9).

In the squid giant axon, the threshold membrane depolarization was found to decrease slightly as the temperature was lowered. The decrease was about 50% for a drop of temperature from 22° to 6°C. When an axon was stimulated by pulses of constant membrane current, a full-sized action potential was developed when the membrane potential reached this critical threshold level. In other words, the threshold membrane potential is practically independent of the intensity and of the duration of the current pulse (in normal axons).

Conduction Velocity. The value of Q_{10} for the conduction rate in the frog myelinated fiber was approximately 1.8 (3, 8). Since a nerve impulse propagates along the fiber as the result of successive electric excitation of the nodes (15), the temperature dependence of the conduction velocity was directly related to the effect of temperature changes upon the latency of the appearance of the action current at the individual nodes (fig. 6). This problem was discussed in the previous section. When a node of a fiber is thrown into action, a 'wave' of a rapid change in membrane potential spreads toward the adjacent node. When the potential at the latter

node is raised to the critical level by this spreading 'wave', an action potential is initiated. The strong temperature dependence of the strength-latency relation discussed above indicates that the major portion of the temperature effect upon the conduction velocity can be explained in terms of the temperature dependence of the resistance of the axis-cylinder and of the nodal membrane. There is at present no direct information about the rate of potential rise at an active node and the relation of this rate to the internodal conduction time.

In the squid giant axon, there is at present no accurate measurement of the temperature coefficient of the conduction velocity. There is little doubt, however, that the coefficient is close to that of the myelinated nerve fiber.

EFFECTS OF PRESSURE CHANGES ON NERVE FIBER

Myelinated Nerve Fiber. The most conspicuous effect of high hydrostatic pressure upon the myelinated nerve fiber was found to be an increase in the duration of the nodal action current. This effect can best be demonstrated by using the experimental set-up of figure 1C. With this method the increase in the temperature of the nerve fiber resulting from compression of the mineral oil in the pressure chamber was minimized by surrounding the fiber with a large volume of Ringer. In figure 7 is presented an example of measurements of the spike duration at different pressures. It can be seen that the duration of nodal activity was increased by a factor of about 4.5 when the pressure was raised from atmospheric pressure up to 10,000 psi. This effect of pressure upon the spike duration was reversible.

The effect of high hydrostatic pressures upon the amplitude of the action current was not marked. At pressures up to about 7000 psi, usually the amplitude increased slightly or remained almost unchanged. At pressures above 8000 psi, the amplitude became slightly smaller than at atmospheric pressure. In a few experiments there was a slight (up to 10%) decrease in the amplitude even below 7000 psi. The conduction velocity was slightly retarded by high pressures. At 5000 psi it was 5–15% lower than at atmospheric pressure. The rheobasic voltage of the fiber was also slightly affected by pressure; at 5000 psi it was increased by 5–20%. When the high pressure was maintained for a long period of time, the rheobasic voltage was found to increase progressively.

Squid Giant Axon. The effects of high pressure upon the squid action potential were similar to those on the frog nerve fiber. At high pressures both the duration of the rising phase and the amplitude of the action potential increased slightly while the duration of the falling phase was increased very markedly. There was a complicating factor, however, in the squid axon. At about 5000 psi the axon started to fire impulses spon-

taneously at a relatively high frequency (up to about 200 per sec.). Because of this spontaneous repetitive firing of impulses, it was difficult to make accurate measurements at high pressures.

Figure 8 shows the effect of a pressure of 4000 psi upon the propagated action potential. Although the shock-response time was also prolonged by

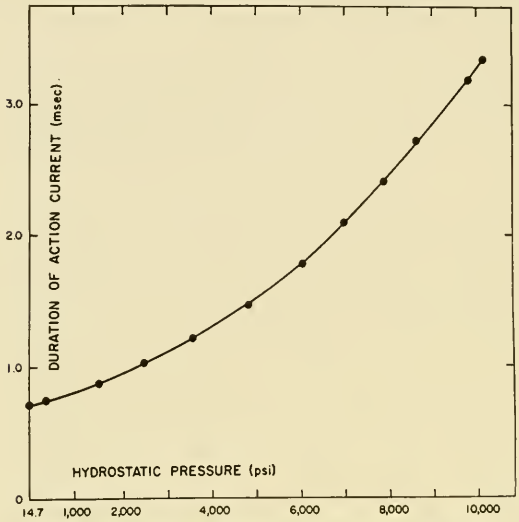


FIG. 7. Effect of pressure on the duration of the action current of the node of Ranvier. Motor nerve fiber of the toad. Temperature 24–25°C.

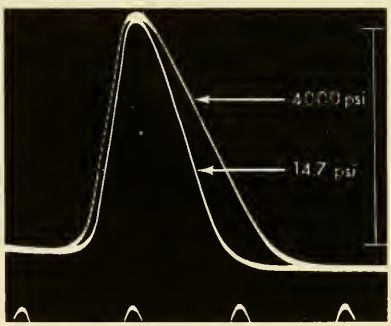


FIG. 8. Effect of pressure upon the action potential of the squid. Vertical bar at right subtends 100 mv. Temperature 23–24°C.

pressure (by 5–15% at 5000 psi) the point of maximum membrane potential of one record was superposed on that of the other record (in order to facilitate comparison of the configurations of the action potentials). It is seen that the rate of potential fall is reduced by about 40% at 4000 psi.

The impedance of the axon membrane was measured with internal electrodes at various pressures. The duration of decreased membrane impedance was found to increase with increasing pressures. The maximum

impedance loss during activity, however, was found to decrease with increasing pressure even though the spike amplitude at the same pressure was unchanged or increased slightly. In many respects the effects of high pressure resembled the effects of low temperature. The effect of pressure upon the impedance loss is illustrated in figure 9.

The resting potential of the squid giant axon was found to be nearly independent of pressure (up to 4000 psi). At pressures higher than 5000 psi, the measurement of the resting potential was disturbed by spontaneous firing of impulses.

The resistance of the resting membrane was found to increase slightly with increasing pressure. At about 5000 psi, the increase was 10–20% of the normal membrane resistance. After exposing the axon to pressures between 9000 and 12000 psi it was frequently observed that the membrane resistance fell to one half to one third of the original value. Upon decompression from the pressures of 9000–12000 psi the recovery of the membrane

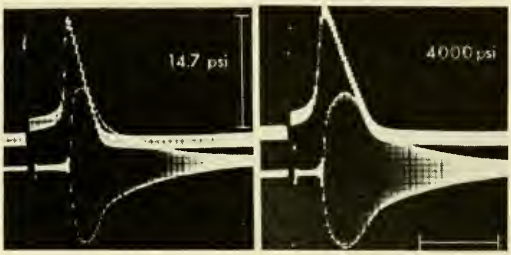


FIG. 9. Effect of pressure upon the simultaneously recorded action potential and the membrane impedance of the squid giant axon. The bridge A.C. was 10 kc/sec. Vertical bar at right subtends 100 mv. Time marking, 4 msec.

resistance was usually poor. The threshold as determined by long rectangular current pulses applied to the membrane through an intracellular electrode was found to decrease very markedly with increasing pressure. At pressures from 3000 to 7000 psi the threshold was reduced to zero and the fiber fired spontaneously.

EFFECTS OF TEMPERATURE AND PRESSURE ON THE NARCOTIZED AXON

It is well known that application of narcotics reduces the height of the action potential, raises the threshold membrane depolarization and decreases the membrane conductance at the peak of activity (see 30). We found in the squid giant axon that these effects of narcosis can be counteracted by cooling or by application of high pressure.

When a giant axon was immersed in a 3% ethanol-sea water solution at room temperature, the spike amplitude recorded with an internal electrode decreased by 30–50%. Frequently the size of the response was reduced to the point where it varied with the stimulus intensity. Under the influence of the narcotic the threshold membrane depolarization was

higher, and the membrane conductance at the peak of activity (measured by the method of voltage clamp) was appreciably smaller than that of the normal fiber. When the temperature of the narcotized axon was changed from 22°C down to 6°–10°C, the spike height recovered to an almost normal value (90–95%). In such a cold ethanol-sea water solution the threshold membrane depolarization usually became smaller than the normal value at room temperature. The membrane conductance at the peak of activity, however, did not change appreciably when a narcotized axon at room temperature was cooled. At very low temperature (3°–5°C), spontaneous firing of impulses was observed in these axons. A similar counteracting ef-

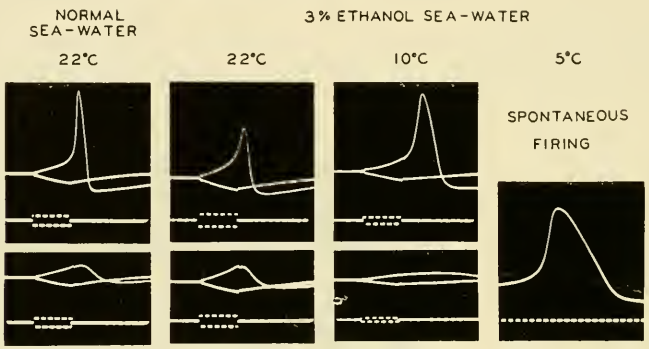


Fig. 10. Effects of temperature upon the spike amplitude and the threshold of a lightly narcotized squid giant axon. The lower records of the three columns at left give a measure of the intensity of membrane current used for threshold excitation. The change in the membrane potential caused by pulses of opposite polarity is also indicated. The effects observed were perfectly reversible. Time marking, 0.2 msec.

fect of cooling was seen with a 5% ethanol-sea water solution. The effects of temperature on the narcotized fiber are illustrated in figure 10.

Application of a high pressure was also effective in partially restoring the spike amplitude reduced by ethanol. The threshold membrane potential and the membrane conductance have not yet been investigated under these conditions.

The effect of temperature and pressure upon the giant axon under ethanol suggests that the action of narcotics upon the axon is analogous to that upon such biochemical systems as the bioluminescent (33). In the bioluminescent system the inhibitory effects of such narcotics as ethanol and urethane can be opposed by pressure. A further investigation into this problem of interaction among narcosis, temperature changes and application of high pressure may throw new light into the mechanism of action potential production.

SUMMARY

The effects of changes in temperature and hydrostatic pressure upon the frog (or toad) single myelinated nerve fiber and the squid giant axon are reviewed. The material presented was obtained partly from observations published by other workers and partly from the authors' recent observations. Special emphasis is placed on the experiments made with internal stimulating and recording electrodes in the squid axon and on the observations carried out with single-node preparations of the toad.

The effects of temperature are discussed with reference to *1*) the resting potential, *2*) the capacity and resistance of the resting membrane, *3*) the resistance of the axoplasm, *4*) the size and shape of the action potential, *5*) the time course of the action current, *6*) the membrane resistance during activity, *7*) thresholds and *8*) the conduction velocity.

The effects of high hydrostatic pressures upon the squid giant axon and upon the toad nerve fiber are discussed and, in particular, a reversible prolongation of the duration of the action potential (up to 4-fold increase at high pressures) at the main feature. A short discussion is given of the temperature and pressure dependence of the effects of narcotics on the nerve fiber.

REFERENCES

1. Lucas, K. The temperature coefficient of the rate of conduction in nerve. *J. Physiol.* 37: 112–121, 1908.
2. Adrian, E. D. The recovery process of excitable tissues. *J. Physiol.* 55: 193–225, 1921.
3. Gasser, H. S. Nerve activity as modified by temperature changes. *Am. J. Physiol.* 97: 254–270, 1931.
4. Bremer, F. and J. Titeca. Etude potentiometrique de la paralysie thermique du nerf. *C. R. Soc. Biol., Paris* 115: 413–417, 1934.
5. Schoepfle, G. M. and J. Erlanger. The action of temperature on the excitability, spike height and configuration, and the refractory period observed in the responses of single medullated nerve fibers. *Am. J. Physiol.* 134: 694–704, 1941.
6. Lorente de No, R. *A Study of Nerve Physiology, 1* and *2*. In: Stud. Rockefeller Inst. Med. Res. 131 and 132, New York, 1947.
7. Lundberg, A. Potassium and the differential thermosensitivity of membrane potential, spike and negative afterpotential in mammalian A and C fibers. *Acta physiol. scandinav.* 15: Supp. 50, 1948.
8. Tasaki, I. and M. Fujita. Action currents of single nerve fibers as modified by temperature changes. *J. Neurophysiol.* 11: 311–315, 1948.
9. Tasaki, I. The excitatory and recovery processes in the nerve fiber as modified by temperature changes. *Biochim. et biophys acta*, 3: 498–509, 1949
10. Tasaki, I. and K. Frank. Measurement of the action potential of myelinated nerve fiber, *Am. J. Physiol.* 182: 572–578, 1955.
11. Cardot, H. and A. Arvanitaki. Les incréments thermiques critiques relatifs aux phases composantes de la réponse électrique oscillatoire locale. Axone isolé de Sepia. *J. Physiol. Pathol. gen.* 38: 9–16, 1941.

12. Hodgkin, A. L. and B. Katz. The effect of temperature on the electrical activity of the giant axon of the squid. *J. Physiol.* 109: 240–249, 1949.
13. Grundfest, H. Effects of hydrostatic pressures upon the excitability, the recovery and the potential sequence of frog nerve. *Cold Spring Harbor Symp. Quant. Biol.* 5: 179–187, 1936.
14. Ebbecke, U. and H. Schaefer. Über den Einfluss hoher Drucke auf den Aktionsstrom von Muskeln und Nerven. *Pflüger's Arch. ges Physiol.* 236: 678–692, 1935.
15. Tasaki, I. *Nervous Transmission.* Springfield, Ill.: Thomas, 1953.
16. Bak, A. A unity gain cathode follower. In preparation.
17. Tasaki, I. New measurements of the capacity and the resistance of the myelin sheath and the nodal membrane of the isolated frog nerve fiber. *Am. J. Physiol.* 181: 639–650, 1955.
18. Cole, K. S. Rectification and inductance in the squid giant axon. *J. Gen. Physiol.* 25: 29–51, 1941.
19. Curtis, H. J. and K. S. Cole. Membrane action potentials from the squid giant axon. *J. Cell. & Comp. Physiol.* 15: 147–157, 1940.
20. Hodgkin, A. L. and A. F. Huxley. Resting and action potentials in single nerve fibers. *J. Physiol.* 104: 176–195, 1945.
21. Hodgkin, A. L. and A. F. Huxley and B. Katz. Measurement of current-voltage relations in the membrane of the giant axon of *Loligo. J. Physiol.* 116: 424–448, 1952.
22. Ling, G. and J. W. Woodbury. Effect of temperature on the membrane potential of frog muscle fibers. *J. Cell. & Comp. Physiol.* 34: 407–412, 1949.
23. Coraboeuf, E. and S. Weidmann. Temperature effects upon the electrical activity of Purkinje fibers. *Helv. physiol. et pharmacol. acta,* 12: 33–41, 1954. In this article reference is made to previous literature.
24. Trautwein, W., U. Gottstein and K. Federschmidt. Der Einfluss der Temperatur auf den Aktionsstrom des excidierten Purkinje-Fadens, gemessen mit einer intracellulären Elektrode. *Pflüger's Arch. ges Physiol.* 258: 243–260, 1953.
25. Tamashige, M. Membrane and sarcoplasm resistance in an isolated frog muscle fiber. *Annotationes Zool. Japan.* 23: 125–130, 1950.
26. Del Castillo, J. and X. Machne. Effect of temperature on the passive electrical properties of the muscle fiber membrane. *J. Physiol.* 120: 431–434, 1953.
27. Hodler, J., R. Stämpfli and I. Tasaki. Über die Wirkung internodaler Abkühlung auf die Erregungsleitung in der isolierten markhaltigen Nervenfaser des Frosches. *Pflüger's Arch. ges. Physiol.* 253: 380–385, 1951.
28. Schmitt, O. H. *Electrochemistry in Biology and Medicine.* Ed. by T. Shedlovsky. New York: Wiley, 1955.
29. Tasaki, I. Initiation and abolition of the action potential of a single node of Ranvier. *J. Gen. Physiol.* 39: 377–395, 1956.
30. Tasaki, I. and K. Mizuguchi. The changes in the electric impedance during activity and the effect of alkaloids and polarization upon the bioelectric processes in the myelinated nerve fiber. *Biochim. et biophys. acta* 3: 484–493, 1949.
31. Tasaki, I. and W. H. Freygang. The parallelism between the action potential, action current and membrane resistance at a node of Ranvier. *J. Gen. Physiol.* 39: 211–223, 1955.
32. Hodler, J., R. Stämpfli and I. Tasaki. Role of potential wave spreading along myelinated nerve fiber in excitation and conduction. *Am. J. Physiol.* 170: 375–389, 1952.
33. Johnson, F. H., H. Eyring and M. J. Polissar. *The Kinetic Basis of Molecular Biology.* New York: Wiley, 1954.

EFFECT OF TEMPERATURE ON VISUAL PROCESSES

V. J. Wulff, *Department of Zoology, Syracuse University, Syracuse, New York*

This paper will be limited to a discussion of the effect of temperature upon the photochemical and electrical events which occur in photoreceptors or their parts.

EFFECT OF TEMPERATURE ON BLEACHING OF RHODOPSIN

One of the temperature-sensitive photochemical processes which has received much study is the bleaching of rhodopsin following illumination. Rhodopsin is the chief vertebrate visual pigment which mediates rod function. It has long been supposed that the light-initiated bleaching of this photosensitive pigment plays a role in the visual act.

Hecht (4) was the first to publish quantitative data on the effect of temperature on the extent of bleaching of extracted frog rhodopsin. His data show that the extent of bleaching is the same at 5.2°C as it is at 2°C and 36°C for a given exposure to light. The Q_{10} of the bleaching process was computed as equal to 1. This temperature independence of the extent of bleaching of rhodopsin reported by Hecht confirmed some earlier observations of Kühne. It should be mentioned here that the bleaching process was not the limiting process in Hecht's experiment. The methodology Hecht employed was to expose rhodopsin solutions at different temperatures to a constant light source for a fixed period of time. The solution was then brought to room temperature in the dark for the density determination. Since the bleaching process proceeds in the dark, it went to completion and was not a limiting process. What the data of Hecht do show is that the rate of excitation of rhodopsin is temperature-independent.

In 1937, Lythgoe (6) first mentioned the occurrence of a color change in illuminated rhodopsin that was cooled to ice temperature. The color produced was orange and, since the orange color faded upon warming, even in the dark, he called the orange state of rhodopsin 'transient orange'. Since Lythgoe's original observation, blocking of the bleaching reaction by low temperatures (i.e. dry ice temperatures) has been demonstrated by Broda and Goodeve (1) and by Wald *et al.* (7); there can be no doubt that the bleaching process is slowed appreciably, if not blocked entirely by low temperatures.

More recently Hagins (3) and Wulff *et al.* (9, 10) have obtained yet additional evidence for the temperature dependence of the bleaching of

rhodopsin. Hagins worked with the excised eyes of albino rabbits, previously dark-adapted, and measured the brightness of a monochromatic light beam reflected out of the eye and into a photomultiplier tube. The visual pigments were excited by a flash of light obtained by discharging energy, stored in capacitors, across a spark gap built into a xenon filled tube. The changing intensity of the reflected monochromatic light beam following the 20 μsec. light flash was displayed on an oscillograph. The

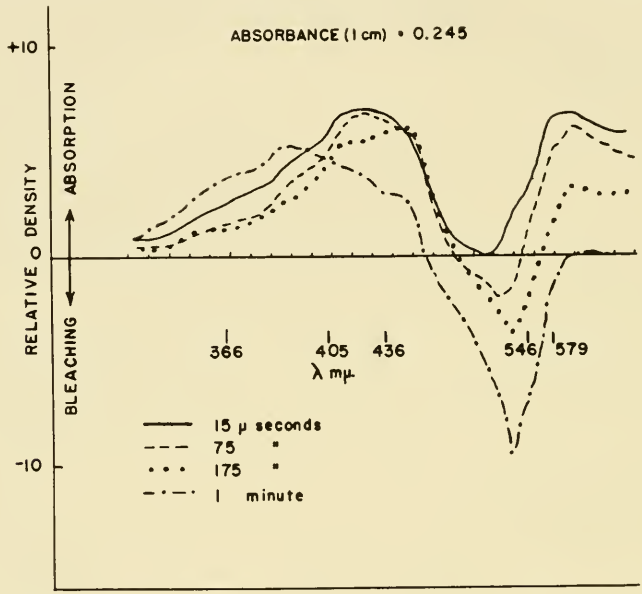


FIG. 1. Difference spectra of degassed rhodopsin solutions after flash excitation in a 5-cm absorption cell. The times indicated are the intervals between beginning of the exciting flash and beginning of the analyzing flash. The wave lengths indicated are obtained from a mercury arc spectrum photographed on the same plate. The ordinate is in relative units; to convert ordinate to actual change in absorbance of the solution, multiply by 0.043.

preliminary results published to date show a detectable reduction in absorption of the reflected monochromatic beam when the wave length is 486 mμ. The reduction in absorption follows a characteristic exponential time course which, at 12°C, has a half-time of 20 msec. and at 26°C has a half-time of 1 msec. Thus, this visual process, which Hagins believes to be the disappearance of the intermediate state metarhodopsin (7), is markedly affected by temperature.

The work in our own laboratory has been with aqueous digitonin solutions of cattle rhodopsin which were subjected to two consecutive flashes; the first flash is a high energy flash (400 joules) to excite the pigment

and the second is a low energy flash (14 joules) to photograph the absorption spectrum on a spectrograph plate. Control spectra were obtained by using an inverted flash sequence, i.e. the low energy analytical flash was fired first and the exciting flash last. The spectra on the plates were then subjected to densitometry and difference spectra were constructed from the comparison of experimental and control spectra. Any one difference spectrum represents density changes in one absorption spectrum when compared to a control spectrum.

Figure 1 illustrates the absorbance changes which occur in rhodopsin solutions in a 5-cm absorption cell during and following flash excitation. During the flash excitation rhodopsin exhibits two new absorption bands, one in the red with a maximum at 600 mμ and one in the blue with a maximum at 425 mμ. These bands are short-lived, having a duration less than 200 μsec. Bleaching of the rhodopsin solution is evident 75 μsec. after onset of the exciting flash and progresses with time, reaching a maximum

TABLE 1. DECREASE IN ABSORBANCE OF RHODOPSIN AT 530 Mμ

Flash Interval	9°C	22°C
msec.		
0.056	0.057	0.333
0.447	0.172	0.532
0.893	0.276	0.732

about 5 msec. after excitation at 28.5°C. The decrease in absorbance of rhodopsin following flash excitation is maximal at 530 mμ. The difference spectrum of rhodopsin an hour after excitation shows a maximal decrease in absorbance at 500 mμ. The discrepancy of 30 mμ in the bleaching maximum may be caused by absorption bands of intermediates in the bleaching process.

Lowering the temperature of the rhodopsin solutions has little effect on the transient absorption bands but markedly slows the bleaching process. Table 1 gives the changes in absorbance of rhodopsin during the first millisecond following flash excitation at 9° and 22°C. Note that both the rate and extent of bleaching is markedly depressed at the lower temperature.

EFFECT OF TEMPERATURE ON RETINAL ACTION POTENTIAL

Temperature also has marked effects upon two characteristics of the photoreceptor response, the latent period and the magnitude of the retinal action potential.

The temperature dependence of the latent period was first demonstrated by Hecht (5) employing the light-initiated siphon withdrawal reflex of

the clam, *Mya arenaria*. Hecht found the duration of the latent period was inversely related to the temperature. The average Q_{10} was 2.5.

More recently, the effect of temperature on the latent period of the retinal electric response has been investigated in the grasshopper, *Limulus*, and frog photoreceptor. The data show that latent period and temperature are reciprocally related. The Q_{10} values obtained range from 2.34 to 4.01, the larger values applying to low temperature ranges. The results from all animals are rather similar and may be illustrated by the data obtained from the grasshopper (fig. 2).

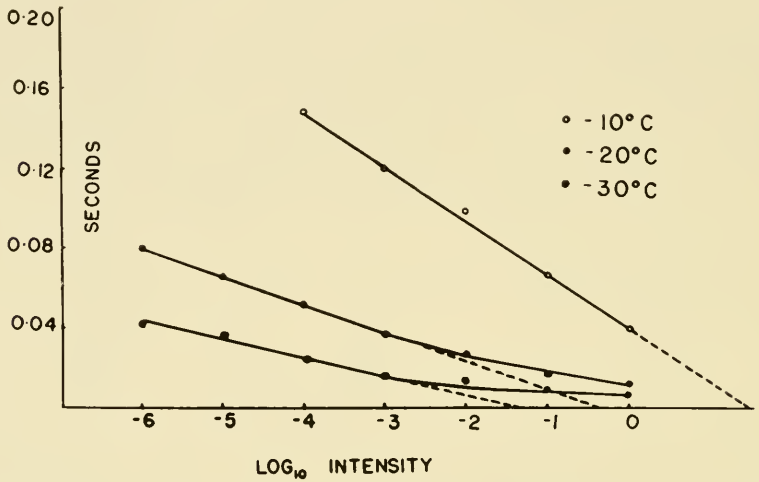


FIG. 2. Curves showing the relationship between the latent period of the retinal action potential and the logarithm of the intensity of illumination, and the effect of temperature on this relation. The data were obtained from the eyes of intact dark-adapted grasshoppers equilibrated to the indicated temperatures for several days. The flash durations used were 0.05 and 0.01 sec. and were maintained constant in any one experiment. Unit intensity represents 11,800 foot candles at the cornea.

The effect of temperature upon the retinal action potential is in sharp contrast to the temperature effect on the latent period. In the grasshopper, it is possible to select intensities and short durations of stimulation such that the magnitude of the response is not at all affected by temperature (fig. 3). On the other hand, one can select long flash durations such that the magnitude of the potential is larger, the lower the temperature. The marked differences in the behavior of the latent period and the magnitude of the retinal action potential to altered temperatures are predicted by a kinetic model of coupling processes between the photochemical and electrical events in photoreceptors (8, 2). Incidentally, the kinetic model was developed independently of the temperature effects just described. The

model assumes two coupling processes between the photochemical and electrical events: an electrochemical process and a timing process which determines when the potential change becomes manifest.

The electrochemical model is defined by the equation $dc/dt = bI - k(c - c_1)$. The positive term controls the formation of an electrochemical substance, C, and the negative term controls the breakdown or disappearance of this substance. The first term is directliy linked to the photochemical event and may be assumed to be temperature insensitive; the second term is a decay process and may be assumed to be temperature

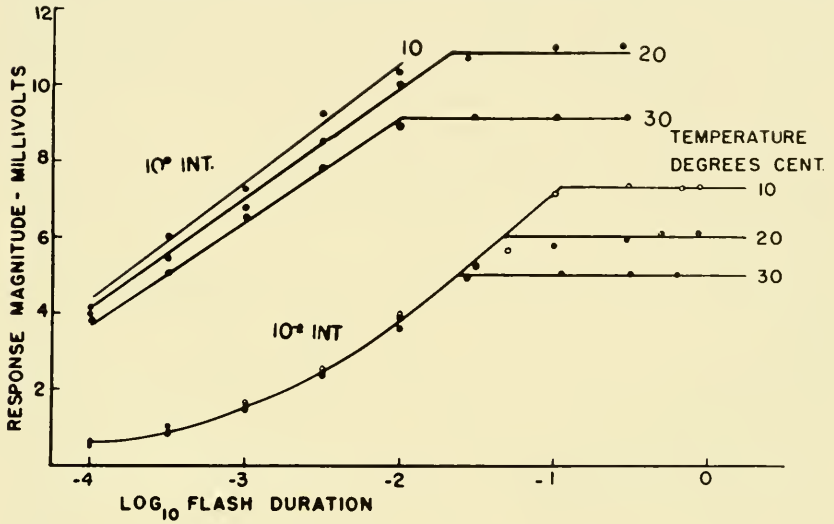


FIG. 3. Curves showing the relationship between the magnitude of the retinal action potential and the logarithm of the duration of illumination, and the effect of temperature on this relation. The data were obtained from the eyes of intact dark-adapted grasshoppers equilibrated to the indicated temperatures for several days, and at two levels of illumination. Unit intensity represents 11,800 foot candles at the cornea.

labile. When stimulating with short light flashes, the first term largely controls the equilibrium concentration of the material, C (11). The model predicts, therefore, that temperature should not appreciably affect the magnitude of the retinal action potential obtained in response to short light flashes. In the grasshopper this is indeed so, and in the case of *Limulus* and frog photoreceptors the effect of temperature is small, Q_{10}'s from 1.0 to 1.3. On the other hand, for longer exposures the decay process contributes more and more to the equilibrium concentration of the electrochemical substance; here temperature should have an effect, but the effect should be inverse, i.e. lowering the temperature should slow the decay process, thus increasing the equilibrium concentration of the electrochemical sub-

stance and, consequently, the magnitude of the retinal action potential (fig. 3).

The effect of temperature on magnitude is not consistent under all conditions of stimulation, nor for all photoreceptors. Both the *Limulus* lateral eye and the frog eye show slight temperature effects on the response magnitude; but even here the effect of temperature on the magnitude of the retinal action potential is much less marked than is the effect of temperature on the latent period.

The model suggested for the latent period is defined by the equation $dp/dt = hp + nI$ and it contains a photochemical term and an autocatalytic term, both positive. These processes cause the accumulation of a factor P to a critical concentration p_c. When this critical level is reached the latent period ends and the electrical response begins. Here again, one may assume that the photochemical term is relatively temperature stable whereas the autocatalytic term is temperature labile; i.e. with increasing temperature the rate of the autocatalytic process speeds up, p_c is reached sooner and the latent period is shortened.

The latency model predicts that the rate constant, h, of the autocatalytic process should increase with increasing temperature. This constant may be evaluated from the data using the expression, $m = -\log 10/h$, and it does, indeed, increase with temperature, exhibiting an average Q_{10} of 1.5. The model permits the evaluation of the ratio p_c/n, from the relation $I_i = hp_c/n$. The quantity p_c/n varies markedly with temperature, decreasing as the temperature increases. Between 10° and 20°C this quantity decreases by a factor of 156 and between 20°C and 30°C it decreases by a factor of 15. Since n is the rate constant of the photochemical process in the latency model, it may be assumed not to vary greatly with temperature. Consequently, the changes in the ratio p_c/n may largely be attributed to changes in the quantity, p_c.

Although it is impossible at this time to draw quantitative relationships between the electrical events in photoreceptors and the photochemical events, it is perhaps of interest to speculate. The bleaching process both in the intact photoreceptor (3) and in rhodopsin solutions (9, 10) is the only transient event thus far observed, which has the time course and temperature sensitivity compatible with the characteristics of the latent period of the retinal action potential. The relative insensitivity to temperature of the transient absorption bands which appear during photoexcitation (9, 10) suggests a possible association with the events leading to the generation of the retinal action potential.

SUMMARY

The effect of temperature upon the photochemical and electrical events which occur in photoreceptors or their parts is discussed on the basis of

previously established and of recently obtained evidence, with especial reference to *1)* changes in the absorption spectrum and the kinetics of the bleaching process of rhodopsin, both in solution and in intact retinas, at temperatures above 0°C (very slow, if any bleaching occurring at temperatures below 0°C), and *2)* the latent period and magnitude of the retinal action potential. With respect to *2*, the latent period becomes shorter with rise in temperature, Q_{10} values having been found to range from 2.3 to 4.0, whereas the magnitude of the action potential is only slightly affected by temperature, the Q_{10} values ranging from 1.0 to 1.3. The effect of temperature on these processes is discussed in terms of a kinetic model of two coupling processes: an electrochemical process generating the retinal action potential, and a timing process controlling the duration of the latent period.

REFERENCES

1. Broda, E. E. and C. F. Goodeve. The behavior of visual purple at low temperature, *Proc. Roy. Soc. London, S B.* 130: 217, 1941.
2. Fry, W. J., V. J. Wulff and M. Brust. Retinal action potential—effect of temperature on magnitude and latency in the grasshopper. *J. Cell. & Comp. Physiol.* 45: 265–272, 1955.
3. Hagins, W. A. Flash photolysis of rhodopsin in the retina. *Nature* 177: 989–990, 1956.
4. Hecht, S. The effect of temperature on the latent period in the photic response of *Mya arenaria*. *J. Gen. Physiol.* 1: 667–685, 1919.
5. Hecht, S. Photochemistry of visual purple. II: The effect of temperature on the bleaching of visual purple by light. *J. Gen. Physiol.* 3: 285–290, 1920.
6. Lythgoe, R. J. The absorption spectra of visual purple and indicator yellow. *J. Physiol.* 89: 331–357, 1937.
7. Wald, G., J. Durell and R. C. C. St. George. The light reaction in the bleaching of rhodopsin. *Science* 111: 179–181, 1950.
8. Wulff, V. J. Annual Progress Report submitted to Office of Naval Research, 1955.
9. Wulff, V. J., E. W. Abrahamson, R. G. Adams and H. Linschitz. Transient spectral changes of rhodopsin and retinene following flash excitation. Unpublished.
10. Wulff, V. J. and R. G. Adams. Annual Progress Report submitted to the Office of Naval Research, January, 1956.
11. Wulff, V. J., W. J. Fry and F. A. Linde. Retinal action potential—theory and experimental results for grasshopper eyes. *J. Cell & Comp. Physiol.* 45: 247–264, 1955.

INFLUENCE OF TEMPERATURE UPON RESISTANCE TO ASPHYXIA[1]

James A. Miller, Jr., *Department of Anatomy, Emory University, Georgia*

Advances in pre- and postnatal care of the mother have so reduced the hazards of childbirth that in many hospitals thousands of babies are born without a maternal death (146). To the baby, however, his birth day has not only remained the most hazardous day of his life but, by virtue of the great improvements in mortality rates for the other 364 days of the first year, is becoming relatively more dangerous than ever before (120). It is well recognized that asphyxia of the newborn is the largest single factor in death during the perinatal period. In addition, the role of asphyxia in the genesis of cerebral palsy and many types of mental defects is beginning to be accorded the attention that is deserves (26, and references quoted). Twelve years ago it occurred to the writer that a new approach to the treatment of asphyxia of the newborn might be made through the aegis of hypothermia, based on the very elemental rationale that a reduction of body temperature should prolong asphyxial survival by reducing metabolic requirements. If the logic held, it should not only postpone the time of death, but actually prevent it. In addition, hypothermia should reduce the brain damage which often makes its tragic appearance in individuals in which resuscitative measures are successful.

THE PROBLEM

The events which lead a person to undertake a research problem are usually of interest and often are instructive in addition. Perhaps it might be of interest to general physiologists to learn that the concept of utilizing hypothermia for asphyxia in man was a direct result of training in and experience with the invertebrates. The author's experience with hypothermia began when as a graduate student it was discovered that reduction of temperature was the safest and most easily controlled method of narcotizing planarians for transplantation operations (115, 116). Thirteen years later the sight of an asphyxiated newborn infant being warmed in a bassinet raised important questions. Why elevate the metabolism and therefore the requirements for oxygen at a time when vital processes are in jeopardy from its lack? Might it not be that this treatment actually

[1] Aided by grants from the National Institutes of Health and Emory University.

shortens life, and that reduction of body temperature in this situation would prolong the period during which resuscitative measures would be effective? Since the Q_{10} of most living processes is between two and three, reduction of 20°C in body temperature might increase anoxic survival by as much as four times. In addition, since the newborn mammal, including man, is essentially poikilothermic (23, 66, 91, 62, and others), problems of activation of homeostatic mechanisms which might arise in the adult would be minimized. Finally, it became obvious early in our experiments that, since these homeostatic mechanisms are completely blocked by anoxia, hypothermia should likewise prove effective in protecting the adult from the effects of asphyxia. The results of experiments designed to test the validity of these concepts constitute the main subject matter of this report. As a result of subsequent extension of the scope of the problem to include other means of reduction of metabolism and attempts to increase energy sources, the latter part of this paper will concern itself with the effects of temperature combined with these agents.

An ideal laboratory animal for this study would be born in large litters at the same stage of development as the human. Since this animal has not come to our attention, it was necessary to compromise. Among the common laboratory animals the mouse, rat, hamster and rabbit are delivered in a far less and the guinea pig in a more advanced stage than the human baby. The guinea pig was selected for intensive study because it is the most sensitive to oxygen lack (71) and presumably any treatment which proved beneficial should be more rather than less effective for man. In addition, the important studies of Windle and of Becker have demonstrated that in this species asphyxia at birth produces the same spectrum of behavioral changes as has been observed in man (185). Their resuscitated animals exhibited a variety of neurological symptoms including spastic and flaccid paralyses, ataxias, tremors, twitchings, epileptiform seizures and somnolence. Histological studies of the brains disclosed edema and multiple capillary hemorrhages which appeared within 2 hours and disappeared within 5 days. Neuronal damage was visible in all animals up to 21 days and permanent structural changes were found in two-thirds of the severely asphyxiated animals (186). Tests of the learning ability of animals with no overt behavioral evidence of permanent damage showed that asphyxiated animals required far more trials to learn a maze and after only 2 weeks' rest had largely forgotten what they had learned (30, 31). Hurder has reported similar results in rats (94–96).

EARLY USES OF HYPOTHERMIA

The earliest use of hypothermia as an analgesic is lost in unrecorded antiquity. Undoubtedly its ability to deaden pain was discovered and

rediscovered many times by inhabitants of the colder climes and possibly it was the first effective treatment for pain. Perhaps the earliest record of the use of snow and ice water as a local analgesic was that of Ibn Sina (Avicenna) in the 11th century (82). In 1646 Severinus (162) reported the use of snow and ice in amputations and Napoleon's able surgeon Baron Larrey rediscovered the value of low temperature in surgery during the disastrous retreat from Moscow in 1812 (c.f. 144). Other ninteenth century users of hypothermia as a local anesthetic include Currie (51), Nunn (131), Arnott (24), Blundell (35), Cooke (44), and Bright (37). The latter considered cold superior to opium, morphine and other narcotic agents.

Evidence of the beneficial effects of hypothermia in asphyxia is scattered but appears early and in toto forms an impressive sum. Spallanzani is reported to have found in 1803 that hibernating bats and marmots were unaffected by four hours' exposure to CO_2; whereas, a bird and rat placed in the same chamber died immediately (c.f. 141).

Among the early studies on hypothermia were experiments on the newborn mammal. In 1824 Edwards (60) reported that newborn kittens required longer to drown in water at 20° than at higher temperatures. Over 100 years later these findings received confirmation (57, 90) and recently have been extended to show that the prolongation of life which occurs in cold water is directly related to the body temperatures of the animals (80).

The modern era dates from the late thirties when two independent groups became interested in depression of body temperature. In 1937 Allen published the first of a series of studies in which he demonstrated the value of local refrigeration both in experimental animals and in patients (5–18, 47, 48). His studies generally have not been accorded the recognition they deserve. In his first publication he demonstrated that no shock or gangrene developed after 48 hours of total ischemia from a tourniquet if the leg were packed in ice. Likewise, loops of intestine remained pink and healthy for 16 hours when ligatured and packed in ice. Likewise, in 1948 based upon his studies he made recommendations for the use of hypothermia in obstetrics which are still years in advance of present practices (17).

At almost the same time as Allen was doing his first experiments, Fay and Henny (65) became interested in low body temperature as a result of observations that the distal portions of the extremities, whose skin temperatures are from 3°–11° below that of the body proper, are not subject to metastases of bone malignancies. Inferring from these observations that neoplasms might require a higher temperature in order to grow they tried 'crymotherapy' (= hypothermic therapy) on a woman with

advanced carcinoma of the cervix in which pain was so severe that chordotomy had been advised. Within 36 hours of treatment with circulating water at 5° to 10°C all pain had disappeared, and at the end of six weeks the lesion was healed and the woman was able to return home. Several months later she died from a brain metastasis. Subsequently, Fay and Henny applied local hypothermia successfully for cancers of the breast, cheek and skin. In 1940 Fay (64) reported that results of generalized hypothermia in more than 100 cancer patients adjudged hopeless cases. Body temperatures were reduced to between 32°C and 23°C and cooling was maintained as long as eight days. Although freedom from pain during the treatment was universal, it was not permanent, and regression of the tumors was not demonstrated in this series. In other reports (70, 181, 178) no evidence was found of tumor regression with similar treatment (134). It was unfortunate that these early attempts to utilize generalized hypothermia were not preceded by a more complete study of the effects of low body temperatures. With the reports of deaths during the induction of hypothermia, its hazards were magnified and acceptance by the medical profession was delayed. Likewise, in attempts to use 'crymotherapy' for the entire body, a fact of importance was overlooked. In the earliest and most successful cases, temperatures from 5° to 10°C were used; however, when generalized hypothermia was employed the lowest temperatures which could be achieved were 20°C higher. It appears possible that the differences in success of the two treatments may well be attributed to these great differences in temperature utilized.

General acceptance of hypothermia as a useful tool in the operating room awaited the studies of Bigelow and co-workers in 1950 (32, 33). Since these provided some of the necessary physiological experimentation which was lacking previously, interest in the field was re-aroused and has been on the increase ever since. They demonstrated that with dogs anesthetized to prevent shivering, oxygen consumption changed in a linear fashion with temperature, decreasing during cooling and increasing during rewarming. At 20°C the dogs' oxygen requirements were found to be only 18% of normal and by extrapolation it was calculated that at temperatures between 10° and 12°C the metabolism would be practically nil. In addition, they demonstrated that circulation could be stopped for 15 minutes and the heart opened with complete recovery. Since then most of the research on hypothermia has been carried on by people interested in cardiac surgery (36, 45, 46, 98, 99, 73, 76, 77, 175–177, 104, 29, 133, 72, 27).

PHYSIOLOGY OF HYPOTHERMIA

Interest generated as a result of military requirements has resulted in a large volume of research upon the effects of temperature variations

upon adult organisms. These are reviewed perennially in the *Annual Review of Physiology*. For many aspects of the subject not covered here the interested reader is directed to chapters entitled 'Temperature Regulation' or 'Heat and Cold' in the following volumes: 1 (1939), 3 (1941), 5 (1943), 6 (1944), 7 (1945), 10 (1948), 12 (1950), 13 (1951), 14 (1952), 17 (1955). A more extended discussion of many aspects of the applications of hypothermic physiology to surgery may be found in Virtue's book (182).

The sharp distinction between the primary and secondary effects of hypothermia must be emphasized. That temperature elevation increases and depression decreases rates of activity has long been recognized for poikilothermic animals, plants, unicellular organisms, and for chemical and physical phenomena in general. This primary effect has sometimes been overlooked in studies on adult mammals, in which the secondary effects resulting from homeostatic mechanisms are so prominent. However, these phenomena are not involved in the case of isolated mammalian organs or tissues. Very small fragments of organs can be cultured in suitable nutrient medium at normal body temperature, but the only method of maintaining the vitality of relatively large masses of tissues or organs is by the use of hypothermia to reduce metabolic requirements. In recent years it has been found that the vitality of tissues and even organs can be preserved for long periods at the temperature of carbon dioxide ice (see below). Since, however, it appears unlikely that intact higher organisms can be so preserved, our present interest is limited to temperatures above the freezing point of water. For all adult organs studied the optimal temperature for maintenance of viable tissues has been found to be between 5° and 0°C. Examples of organs which have been kept viable for long periods at these temperatures include skin (42, 19, 143 *et al.*), blood vessels (145), corneas (28, 160, and others), and entire frog eyes (174).

Certain pertinent aspects of the responses of intact animals to low temperature are discussed below.

Metabolism. The fundamental and most important effect of hypothermia is its influence upon metabolism. In mammals as well as in poikilothermic organisms cooling produces a progressive reduction in rate of vital processes in accordance with van't Hoff's law. In the conscious adult mammal reduction of core temperature is resisted by homeostatic mechanisms which raise metabolism about 200–400% (32). Likewise, in newborn guinea pigs we have found increases up to 241% during shivering. When homeostatic mechanisms are blocked by narcosis, and/or ganglioplegic drugs, or by asphyxia, a smooth fall in body temperature with corresponding fall in overall metabolism can be achieved even in adults (32, 59). Bigelow found the metabolism of a dog reduced to 55% at 28°C, 39% at 25°C and

18% at 20°C. Similar findings have been reported by others. Gollan was unable to detect any A-V difference in oxygen saturation at 4°C (72). As a result of the finding that as much as four volumes per cent of oxygen is dissolved in the blood at 3°C he calculated that a dog could be maintained without hemoglobin at temperatures below 10°C. He subsequently demonstrated that such was the case by removing the red blood corpuscles and circulating plasma only (75).

This reduction in metabolism is an over-all average and reduction in some organs may be greater than others. Metabolism studies are needed for organs such as the liver and kidney in which histological damage has been reported following hypothermia of two and a half hours' duration (see below).

Since the brain is the critical organ in asphyxia, the fact that its metabolism is depressed during hypothermia is important (67, 184). More recently a linear fall in oxygen uptake during cooling has been demonstrated. The rate at 26°C was found to be only one third of that at 35°C (157). An independent study reported a three-fold increase in resistance to occlusion of the arterial supply to the brain in dogs at 26°C as compared with those at 35°C (111).

Heart Action. The heart, which appears to be the critical organ in hypothermia, likewise exhibits a regular decrease in oxygen uptake. This has been interpreted by some as a consequence of the decreased coronary flow which also occurs in hypothermia (61). However, since the A-V difference in the hypothermic heart is within normal limits (85, 86, 25), it appears reasonable to attribute the reduced metabolism to the direct action of the cold upon the myocardium. Both embryonal (62, 43) and adult hearts (72, 20–22) can be cooled until all activity ceases and rewarmed with the resumption of normal contractions.

Increased irritability of cardiac muscle as the temperature falls is the most serious secondary effect of hypothermia. This results in atrial fibrillation at about 29°C, followed by ventricular fibrillation at a slightly lower temperature in untreated animals. In recent studies with heart-lung preparations it was found that hypothermia reduces heart rate, but over a wide range of temperatures the maximum stroke volume remains high. Although work capacity decreases it remains well above the work load that the heart must cope with at the same temperature. No relationship was found between previous work load and the onset of fibrillation (155).

Reduction in conductivity during cooling has been demonstrated in man (58, 89) as well as in animals (142, 84). This apparently results in the escape of some areas of the myocardium at low temperatures with the resultant development of ectopic beats. However, another inter-

pretation has been offered. This is the concept that circus movements rather than ectopic foci are responsible for fibrillation (154).

According to Gollan, the tendency to develop ectopic foci is aggravated by increased pressure in the coronary system which causes ischemic anoxia in the myocardium (76). This explanation may perhaps reconcile the contradictory reports on the question whether or not myocardial hypoxia plays a role in ventricular fibrillation. Those who find no evidence that it does include Hegnauer and D'Amato (86), Badeer (25), Reissman and Kapoor (155). Those who consider that hypoxia is important include Bigelow *et al.* (32), Swan *et al.* (177) and Ramos (154).

Vascular Effects. Hypothermia prolongs bleeding and clotting time and reduces circulating plasma volume 10%–12% (52, 164). The fate of the lost fluid is problematical, but it is the cause for the hemoconcentration and for the large amount of fluids which profoundly hypothermic animals require to maintain blood volume and fluidity (78). The great increase in blood viscosity caused by low temperature (84) is a serious problem at temperatures below 20°C. Laufman (103) mentions that the thick consistency of the blood of his patient at 18°C rectal temperature interfered with the taking of samples. Extreme peripheral vasconstriction is a well-known effect of exposure to low temperature. In addition, constriction of deeply placed vessels occurs when cooled blood is returned to the abdominal vessels (106). This causes renal ischemia and anuria, reduced blood supply to the heart and liver (39) and other organs. This may become a serious problem if hypothermia is prolonged in the moderately low temperature range, between 25° and 30°C, in which metabolism may be as high as 50% of normal. At 10°C or less, the oxygen requirements are so low that organs can tolerate ischemia for extended periods.

Respiration. Respiratory rate and excursion decrease with decreasing body temperature and in animals anesthetized with pentothal or Nembutal artificial respiration is necessary below about 28°C (36). On the other hand, with ether respiratory movements continue until after cardiac arrest in most of the cases (85). Respiratory movements of etherized monkeys continue to 20° (41). Since respiratory acidosis has been demonstrated and has been found to reduce survivals, hyperventilation is generally employed (176). Premedication with sodium bicarbonate also has been found to be beneficial (132). Investigators are generally agreed that maintenance of high blood pH gives better survivals (68).

Effects on the Embryo. Since our interest in hypothermia is primarily as a means of combating asphyxia of the newborn, observations with embryonal tissues are of significance. These show that temperatures near zero are not merely tolerated; they are superior to higher tem-

peratures for maintaining the vitality when such tissues have been isolated and thereby deprived of their blood supply. In 1931 Bucciante (40) reported that chick organs in Ringer's solution at 5°C survived for long periods. Skin lived 21 days and even the most sensitive organ, the liver, survived for 3 days. Hetherington and Craig (88) kept chick heart muscle at 0°C for 15 days without apparent effect on vitality. Waterman (183) tested rabbit and chick tissues and organs and determined that 5°C was optimal for survival. At this temperature brain survived several days, intestine for a longer period and skin for 3 weeks. Pomerat and Lewis (152) found newborn human tissues slightly less resistant to the effects of cooling than the corresponding tissues of the chick. The assumption from this line of experimentation is that temperatures in the vicinity of 5°C are probably optimal for survival of avascular or anoxic newborn human organs.

The effects of hypothermia upon the intact newborn animal have been but little studied. Fairfield (62) found that newborn rats made no attempts to control temperature and at 3 days of age produced only a transient rise in metabolism. During cooling the metabolic rate fell with temperature and at 3°C was immeasurably small. She reports complete recovery of an animal which remained apneic for 108 minutes. Electrocardiographic studies show that the infant heart reacts in a similar fashion to that of the adult. Interference with conduction, irregular QRS impulses, the development of 2:1 or 3:1 heart block and occasional fibrillation were observed. In spite of these effects one infant recovered after 82 minutes of asystole.

HISTOLOGICAL STUDIES

Studies of tissues of refrigerated appendages subjected to temperatures in the neighborhood of 0°C show no evidence of damage to nerves, blood vessels, glands and skin (161). Generalized hypothermia, likewise, has been reported to produce little or no change from the normal microscopic appearance of tissues (179, 56). However, recently Krocker (100) reported conflicting results from a careful study of livers, kidneys and adrenals of dogs exposed to hypothermia for 2½ hours. She found depletion of glycogen in the liver, vaculolation of cells in the adrenal cortex, tubular damage in the kidneys and accumulations of fat droplets in all three organs. Other reports of tissue damage resulting from hypothermia include vacuolation of cells of adrenal cortex and medulla (173), kidney lesions, chiefly in distal convoluted tubules (105), reduction of urinary output (158), associated with renal cortical ischemia (130). These changes are in sharp contrast with the behavior of isolated tissues

and organs described earlier. It appears likely that the differences are due to two facts: *1)* the temperatures currently used in hypothermia studies (25°–30°C) are sufficiently high to permit appreciable metabolic activity in organs such as the liver and kidney. Bigelow *et al.* (33) found a residual metabolism of 56% in a dog at 28°C and it is possible that the metabolism of organs such as the liver, kidney and adrenals may not fall as rapidly as the body as a whole. *2)* Because of vasoconstriction, hypotension, and reduced cardiac output, such structures may suffer from increasing anoxia as the period of cooling is prolonged. In the course of 2½ hours at this temperature (the time used by Miss Krocker) serious damage can occur. Evidence in favor of this interpretation is found in the experiments of Falkmer and Kjellgren (63) who by the use of intra-abdominal coils cooled the liver 5° to 10° lower than the rest of the body. In their histological studies only very mild liver injury was found and restitution appeared to be complete within 24 hours. This might also explain the fact that tissue damage has not been demonstrated in limbs in which temperatures approaching 0°C were induced prior to amputation.

That there is nothing inherently dangerous to organs such as the liver, adrenals and kidneys from low temperatures is illustrated by studies on deep hypothermia by the group at the National Institute for Medical Research in London. With the aid of glycerol and −79°C they have developed methods of preserving both motility and fertility of spermatozoa for long periods (135–137, 151, 172, 147–150, 159). With a modification of this technique, red blood corpuscles can be frozen, stored for months and reinjected (165–168, 128, 129, 107–110). Even more remarkable have been the demonstrations that ovaries and testes not only do not disintegrate at −79°C, but can be shown to secrete appropriate hormones upon reimplantation after long sojourns at −79°C outside the body (138–140, 169–171, 53–55). In the case of the testis, active grafts were obtained after 227 days and if implanted into the scrotum produced motile spermatoza (54). Ovaries stored for one year at −190°C still retained capacity to form functional grafts (139). Other applications of their technique for longtime storage of vital tissues include skin (400 days with subsequent successful grafting—ref. 34), arteries (93, 92) and corneas (156). From these facts the impression is gained that the temperature range which is currently employed in generalized hypothermia probably will eventually be included in the so-called 'danger zone'. When the causes of ventricular fibrillation are better understood and controlled, it appears likely that the range between 10° and 0°C will be utilized. As noted above, in this range metabolic requirements approach zero and long periods of total interruption of blood supply or complete tissue anoxia can be tolerated.

FACTORS AFFECTING TOLERANCE OF HYPOTHERMIA

Below is a list of factors known to influence the lower limits of survival under hypothermia.

Age. In all species studied thus far, the resistance to hypothermia is greater in the newborn than adult of the same species (60, 163, 38, 62, 1, 2). These studies include the hamster, rat, rabbit, cat, dog and guinea pig. In table 1 the lethal temperatures for non-narcotized adults and neonatal infants of seven species of mammals are listed. As may be seen, there are no figures for pups and human babies, but recoveries from

TABLE 1. LIMITS OF TOLERANCE OF HYPOTHERMIA IN NONNARCOTIZED ANIMALS

Species	ADULTS		NEWBORN	
	Lethal Temp.	Investigator	Lethal Temp.	Investigator
Hamster	3.8°	Adolph, Lawrow '51	below 1.0°	Adolph, '51
Rat	15.1°	Adolph '48	below 1.0°	Fairfield '48 Adolph '48
Rabbit	17–20°	Frank '07 Jackson, Alonge '34	below 6°	Adolph '51
Cat	19°	Simpson, Herring '05 Britton '22 Meidinger '40	7°–8°	Adolph '51
Guinea pig	17°	Gosselin '49	15° 10°–12°	Adolph '51 Miller, Miller '54
Dog	28°	Woodruff '41 Boerema *et al.* '51	? (16.8° with recovery)	Miller '57
Man	26–27°	Rascher '42 (in Alexander '45)	? (18° with recovery)	Klein '36

temperatures well below 20° are known. Unless the human baby is an exception to the rule, the margin of safety for the use of cooling should be greater than it is in the case of adults. Since in the operating room poor risk patients can be safely cooled to 25°C (177), the probability is that temperatures considerably lower are safe for the newborn.

Narcosis. This factor, which is just beginning to be appreciated (121–124), explains many of the discrepancies in the results of different investigators. For example, from the experiments at Dachau, one would conclude that lethal temperatures for man are between 26° and 27°C, with an occasional individual who can tolerate slightly lower temperatures. In these experiments, the lowest temperature from which recovery occurred was 25.2°C (4). By contrast, Fay (64), who used hypothermia in combi-

nation with sedation, carried his patients down to a minimum of 23.9°C; and Talbott (178) to 23.3°C. Although the subjects in the Dachau experiments were at least partially starved, they certainly were in as good condition as Fay's and Talbott's patients, who were in the terminal stages of cancer. Likewise, the thoracic surgeons using hypothermia for cardiac operations employ temperatures in the neighborhood of 26°C (104, 58, 177). In their operations, temperatures as low as 22°C have been followed by complete recovery (175, 27). The lowest temperature from which recovery has been reported (16°C) was that of a woman who, previous to exposure to low temperature, had been at least partially narcotized by alcohol (103).

Artificial Respiration. Since the commonly employed range for hypothermia is below that at which respirations cease, artificial respiration is necesary and is routinely employed. Both Bigelow and Swan emphasize the importance of hyperventilation as a means of preventing the accumulation of CO_2 in the blood.

Fluid. Hypothermia causes hemoconcentration. As temperatures below 20°C are reached, blood viscosity increases greatly. In Laufman's patient (103) he noted that blood in the patient's femoral vein was completely congealed at the time of admission to the hospital. To avoid both the hemoconcentration and the excessively high viscosity of cold blood, Gollan dilutes it with Ringer's solution until a hematocrit of about 25% is reached. Part of his success in carrying dogs to 0°C with recovery may be attributed to his liberal use of blood diluents.

Ventricular Fibrillation. Many approaches to this problem have been used and with varying degrees of success. Electric defibrillation is by no means infallible. Epinephrine given to stimulate failing hearts was found to increase fibrillation (45, 46). The group at Denver (175, 176) at first used hyperventilation combined with potassium chloride injections to stop fibrillation and calcium to aid in starting the heart again. Later, they found that prostigmine was effective in preventing ventricular fibrillation in dogs and have been using it routinely as a prophylaxis (153). They have performed a large number of operations with a very low incidence of death from fibrillation (177).

People working with pump oxygenators and extracorporeal cooling report very little difficulty with ventricular fibrillation. Juvenelle has permitted fibrillation to continue five hours in a dog and upon rewarming regular effective beats recommenced. Gollan uses premedication with quinidine sulphate (113, 56) to prevent fibrillation during the rewarming process. He emphasizes also the importance of reducing coronary flow to prevent cardiac dilatation and pressure ischemia of the myocardium which he believes contributes to the tendency of the cold heart to fibrillate (78).

HYPOTHERMIA AND ASPHYXIAL SURVIVAL

As a result of studies on newborn rats and puppies, Himwich concluded that poikilothermia is an important factor in the high degree of resistance to anoxia shown by mammalian fetuses (91, 66). He did not, however, suggest the trial of hypothermia as a means of increasing asphyxial resistance. Neither did van Harreveld and Tyler (83) even though they demonstrated that in cats there was less injury to the spinal cord after 120 minutes of asphyxia at 27° than after only 35 minutes at normal temperatures.

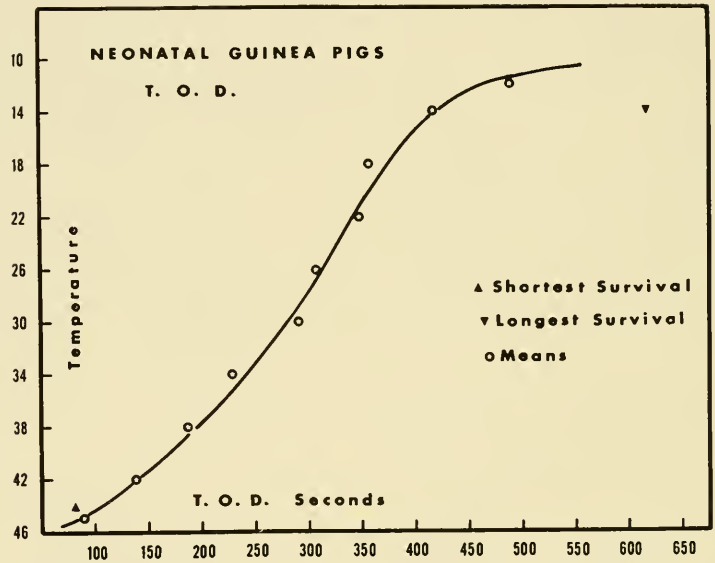


FIG. 1. Time of death of 205 neonatal guinea pigs exposed to 95% N_2 + 5% CO_2 at different body temperatures. Data from table 1, Miller and Miller, 1954.

In our early experiments it was found that the time of death (T.O.D.) of newborn guinea pigs exposed to 95% N_2 + 5% CO_2 was increased by reduction of body temperature and that cooled animals could recover completely from exposures which were lethal for warmer littermates (117). Since the implications of these findings were completely contrary to standard practice for the treatment of shock and asphyxia of the newborn, more extensive studies were made with hundreds of animals (127, 121, 122). The temperature range of the T.O.D. experiments was extended from lethal heat to lethal cold in a series of 205 animals exposed until death. In order not to complicate problems of interpretation, no attempts were made in this study to counteract these secondary effects of hypothermia, even though it was obvious that many of the animals that died

could have been saved by artificial respiration and various other treatments.

Figure 1[2] shows that in the range from 43° to 20°C survival time increases approximately 50% per 10° decrease in body temperature. Below 20°C cooling is increasingly effective on the average, in spite of the fact that morbidity from hypothermia was obvious in a number of the animals (c.f. 122, 119). As temperatures were reduced further animals actually died from secondary effects of low temperature before exposure.

The time of death and temperature of the longest and shortest-lived animals are also illustrated on the graph. The longest survival (617 seconds) was that of a 14°C animal. It lived more than 7½ times as long as the shortest-lived animal (81 seconds) whose body temperature was 42.8°C. The 10 shortest-lived animals with a mean temperature of 42.8°C averaged 95 seconds survival. The 10 longest-lived animals, with a mean body temperature of 14.9°C, averaged 548.9 seconds (120). These

Table 2. Cooling during asphyxiation*

CONTROL		COOLING BEGUN AFTER LOSS OF EQUILIBRIUM		
Max. Temp. Drop	T.O.D. (after fall-over)	Max. Temp. Drop	T.O.D. (after fall-over)	% of Control
1.8°	185.8 sec.	11.9°	214.3 sec.	114.9% ± 2.9

* Condensation of table 2, Miller and Miller, 1954.

experiments prove that hypothermia is effective in delaying asphyxial death when induced before asphyxiation. In a second series of experiments, it was found that when cooling was begun after loss of consciousness from asphyxia, a significant increase in survival time could still be demonstrated (table 2).

The effect of temperature upon asphyxial survival has been tested on two species which are less rather than more mature at birth than man. In figure 2 is plotted the time of death and temperature of 18 newborn rabbits. Littermates are connected by lines. It shows that not only are rabbits far more resistant to asphyxia than guinea pigs at normal body temperature, but also that the protection against asphyxia conferred upon them by cooling is almost three times that observed in the guinea pig. Figure 3 shows the times of death of a litter of six neonatal puppies at various body temperatures. Hypothermia is even more effective here than in the newborn rabbit in protecting against asphyxial death. From general considerations one might expect the human baby to react more

[2] Curve drawn from 4°C means published in table 1, Miller and Miller (122).

like the pup than the baby guinea pig, rabbit or rat (62), but at present there are no data available on any primate.

Postponement of the time of death does not necessarily indicate that recovery from a lethal exposure will likewise take place. To test this possibility, 100 animals, including 10 litters of 4 and 30 littermate pairs, were exposed to asphyxia. Table 3 shows that all animals at 30°, 25° or 20°C recovered completely from 15 seconds more than the lethal exposure

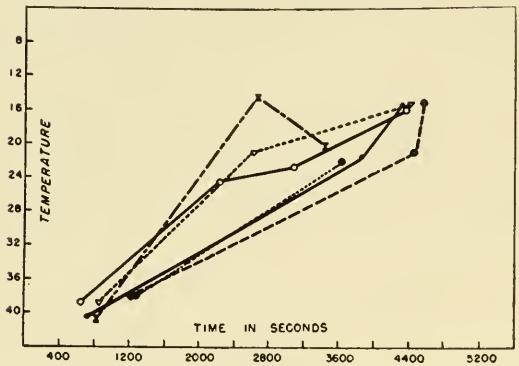


FIG. 2. Time of asphyxial death of 18 neonatal rabbits exposed to 95% N_2 + 5% CO_2 at different body temperatures. Littermates are indicated by lines connecting the points.

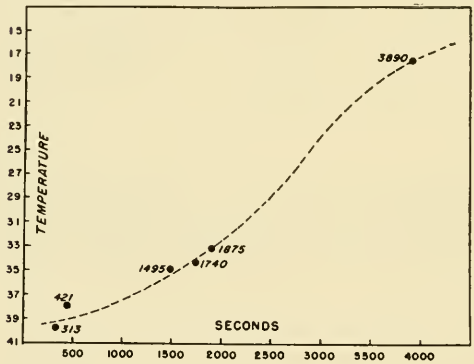


FIG. 3. Time of asphyxial death of a litter of neonatal puppies exposed to 95% N_2 + 5% CO_2 at different body temperatures.

for their 40°C littermates. Likewise, all animals at 25°C and almost half of those at 20°C recovered from 15 seconds more than the lethal exposure for littermates at 35°C and 30°C respectively.

Without measures to combat the secondary effects of hypothermia, temperatures from 20°C downward become hazardous. It should be emphasized, however, that every cooled animal above 20°C recovered from more than a lethal exposure for its warmer littermate. The author feels justified in concluding that the criticism that hypothermia can "postpone asphyxial death but does not prevent it" is refuted by the results of these controlled experiments.

In a limited series (20 experimentals and 20 controls), animals were cooled to 20°C, severely asphyxiated, and the effects of rapid rewarming in a water bath at 45°C were compared with spontaneous recovery. Of those that survived, the rewarmed animals reached each stage of recovery in about half of the time required by the controls. However, since eight rewarmed and nine controls failed to recover, it was concluded that recovery from asphyxia in cooled animals depends largely upon the length and severity of the asphyxia and that events during the subequent recovery period are of lesser significance.

Measurements of oxygen uptake before and after cooling gave a mean depression of 56.8% per 20°C depression of body temperature. This is in good agreement with expectation of a 50% depression if the asphyxial

Table 3. Percentage recovery from asphyxia*

	40°	35°	30°	25°	20°	15°
Series 1 (5 litters of 4)	0.0%		100%	100%	100%	
Series 2 (5 litters of 4)		0.0%		100%	40%†	0.0%
Series 3 (30 pairs)		0.0%	0.0%	100% 0.0%	47%†	0.0%†

* Condensation of table 5, Miller and Miller, 1954.

† Mean survival time for animals which died was greater than that of controls.

protection conferred depends entirely upon reduction in metabolic requirements (120).

Experiments with 300-gram guinea pigs showed that, although it is not as effective as in the newborn, hypothermia prolongs asphyxial survival in young adults as well (118, 127). Figure 4 based on the time of asphyxial death (T.O.D.) of 197 animals shows that temperatures below 20°C become increasingly hazardous on the average. However, it is to be noted that the longest survival in the series was an animal at 15.4°C who lived 286 seconds. This suggests that in the case of adults as well as the newborn, it is the secondary effects of hypothermia which alter the picture below 20°C. As a corollary it may be predicted that the maximal hypothermic protection against asphyxia for adults will probably be found close to 0°C when secondary effects of low temperature are controlled. As in the case of the newborn, adult animals cooled 10° or 15°C recover

from an exposure to asphyxia which is lethal for controls at normal body temperature.

Since perhaps the most critical factor in recovery from shock is tissue anoxia which occurs as a result of the cardiovascular failure, it is interesting to note that successful use of hypothermia in treatment of shock in humans has been reported by a number of workers (12, 17, 18, 49, 50, 101, 102).

If hypothermia prolongs asphyxial survival because it reduces the organism's need for oxygen, other treatments which reduce metabolic requirements should have a similar sparing action in asphyxia. This was found to be the case with sodium pentobarbital. Preliminary tests

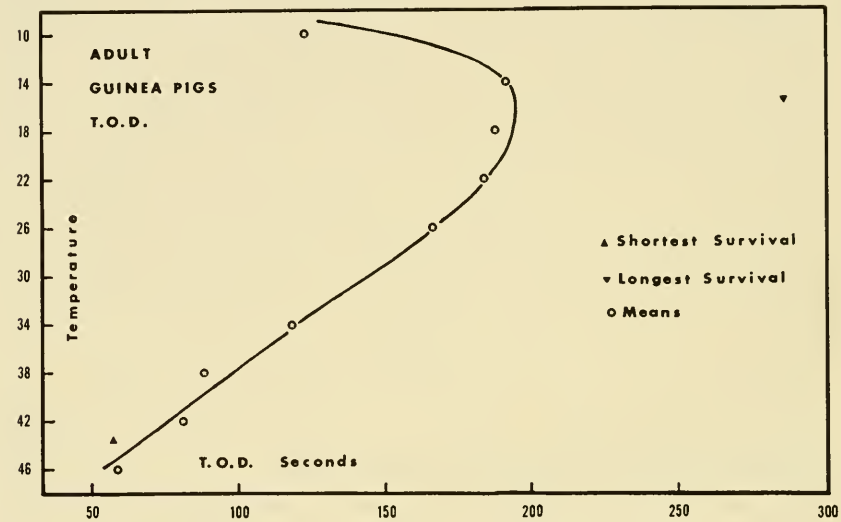


FIG. 4. Time of death of 197 young adult guinea pigs exposed to 95% N_2 + 5% CO_2 at different body temperatures.

made at four temperatures within a range of 10°C showed that survival times were longer than normal in the narcotized animals at all four temperatures. In addition, it was noted that the survival times of the coolest narcotized animals were twice those of the warmest. This is an increase of 100% per 10°C decrease in temperature instead of 50% as had previously been found for non-narcotized animals. Next, the temperature range was increased and littermate pairs were tested at seven temperatures over a range of 25°C (fig. 5). This confirmed the fact that the beneficial effects of narcosis in asphyxia increase as the temperature decreases. At 15°C the Nembutal-injected animals lived twice as long as their water-injected littermates. Recovery of narcotized animals from an exposure which was lethal for non-narcotized littermates was demon-

strated and it was possible to show that the majority of narcotized animals cooled below 20°C could recover from two times the lethal exposure for

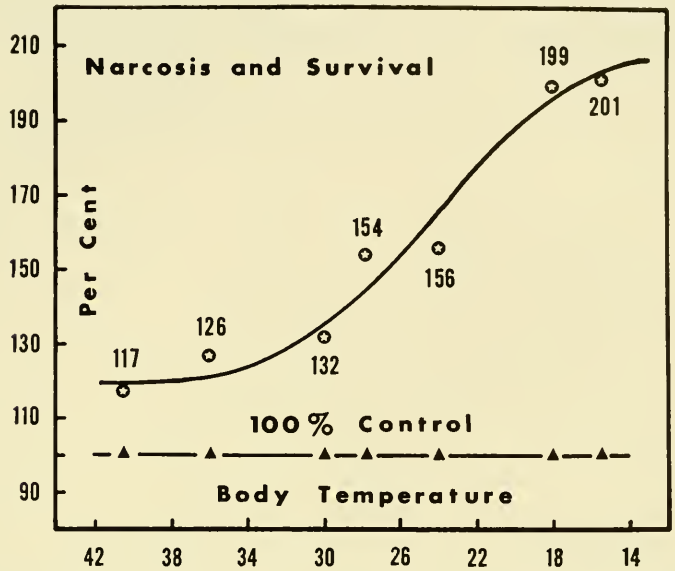


FIG. 5. Effect of narcosis upon time of death of neonatal guinea pigs at seven different body temperatures. Results graphed as percentages of non-narcotized littermate survival times. Data from Miller and Miller, 1957.

TABLE 4. CONTROL AT NORMAL TEMPERATURE NARCOTIZED, COOLED LITTERMATE GIVEN TWO TIMES LETHAL EXPOSURE*

	TEMP.	T.O.D.	EXPOSURE TO ASPHYXIA	DIED	RECOVERED	
					Immediate	24 hour
	°C	sec				
H_2O control	37.7	211.4 sec.		10	0	0
Narcotized, cooled littermate	19.3		422.8 sec.	1†	9†	7

* From table 4, Miller and Miller, 1957.

† Two additional animals died during the subsequent 24 hours.

littermates at normal body temperature. Table 4 shows 90% immediate recoveries of 19°C animals; 70%, 24-hour recoveries.

To test whether struggling and shivering during the induction of hypothermia influence subsequent resistance to anoxia, five littermate pairs were cooled to approximately 24°C. One member of each pair was injected with Nembutal before cooling, the other after the desired temperature

was reached. Table 5 shows no difference in the survival times of the two groups.

Electrocardiograms have been made on 13 animals during asphyxiation. A preliminary study of these indicates that although the time of last gasp was delayed, the time of ventricular arrest was not affected by pento-

Table 5. Cooling before and after narcosis*

	TEMP.	T.O.D.,	%
	°C.	sec.	
Cooling after narcosis	23.6	547	100
Cooling before narcosis	23.7	555	102

*Condensed from table 5 Miller and Miller, 1957.

Table 6. Effects of temperature and sedation on time of ventricular arrest

	TEMP.	LAST GASP	VENTRIC. ARREST
	°C	sec.	sec.
Normothermic	36.8	272.8	809
Sedated normothermic	37.3	530.5	940
Hypothermic	21.9	399.3	1570
Sedated hypothermic	20.1	674.5	1500

Table 7. Effects of narcosis on oxygen uptake and on asphyxial survival

	REDUCTION OF OXYGEN UPTAKE	PROLONGATION OF ASPHYXIAL SURVIVAL
Normothermic	−17.5%* (approx. 39°C)	+17.1% ± 8.9† (approx. 40°C)
Hypothermic	−49.7%* (approx. 21°C)	+52.2% ± 9.7† (approx. 24°C)

* From table 9, Miller and Miller, 1957.
† From table 2, Miller and Miller, 1957.

barbital in either normothermic or hypothermic animals (table 6). If confirmed by further studies, these findings indicate that the heart is not benefited by sedation during asphyxia.

Oxygen uptake measurements were made of narcotized animals before and after cooling. These agreed well with the previously observed mean prolongation of asphyxial survival by narcosis in the two temperatures measured (table 7). This finding indicates that the protective action

of sodium pentobarbital injections in asphyxia is due largely to the depression of metabolism which it produces.

Tripp's successful use of H_2O_2 in treating experimental anaerobic peritonitis in guinea pigs (180) suggested the possibility of its use as a source of oxygen in asphyxia. Tests showed that 2½ cc/100 gm of 3% peroxide injected intraperitoneally increased survival time, shortened recovery times for sublethal exposures and was more effective than similarly injected oxygen (125). Because occasionally animals showed transient symptoms of gas emboli in the brain, 5 cc/100 gm of 1% solutions have been used more recently. These have been effective in prolonging the time of death, shortening recovery time, and in preventing death from exposures lethal for control littermates. Although experiments to test whether hypothermia potentiates the protection against asphyxia provided by peroxide are incomplete, the data already at hand indicate that such is the case.

Adenosine triphosphate is a high energy nucleotide and plays an important role in tissue metabolism. On the other hand it has been shown to be reduced markedly in experimental anoxia (3) and is known to require oxygen for resynthesis. Accordingly, tests were made of the effects of exogenous ATP upon resistance to asphyxia. T.O.D. experiments showed only a small increase over controls (21%), but this protection was sufficient to permit recovery of 100% of the ATP animals from an exposure which was lethal for littermate controls. In the light of these findings we were unprepared for the magnitude of the reduction when sublethal exposures were tried and stages of recovery were timed. Figure 6 shows that the mean times of first breath, attempt to right (RR Attempt), completion of righting (Compl. RR) and crawl were well below that of the controls. The righting reflex is the most consistent of the stages timed. It is entirely spontaneous, and once the animal has gotten upon its feet it will not permit itself to be placed on its side. In these experiments the ATP animals required less than half of the time (46% ± 2.4) of the controls to reach this stage. Comparisons of the activity of ATP, ADP and AMP show that the original rationale for its use is untenable. However, data of Green and Stoner (81) on the effects on metabolism of injected ATP and AMP suggest that here too general depression of metabolic requirements may be the mechanism of the beneficial effects of the nucleotides (120). Effects of hypothermia on nucleotide protection against asphyxia are being investigated and metabolic tests will also be made to see if the newborn guinea pig shows the same reaction to injected nucleotides as the adult rat used by Green and Stoner.

It cannot be predicted *a priori* whether measures which are individually effective will be additive when they are combined. A beginning has been made in testing the combination of two or more treatments. Table 8

shows that injections of 5 cc of water per 100-gram body weight increased asphyxial survival 24%, a fact which has been noted in many experiments but upon which no comments have been made until now. Litter-

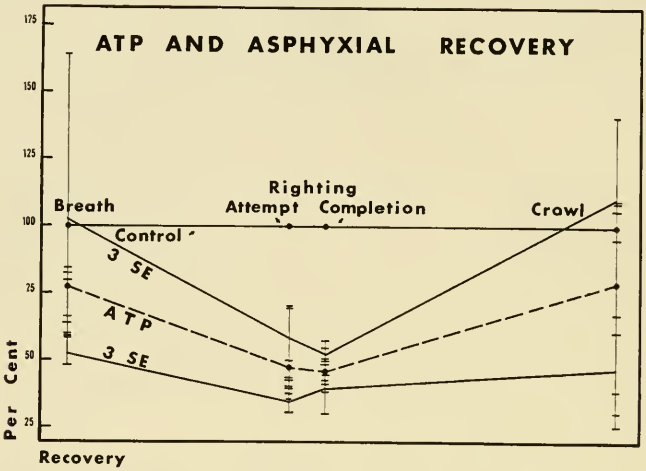


FIG. 6. Effects in neonatal guinea pigs of intraperitoneal ATP upon recovery from sublethal exposures to 95% N_2 + 5% CO_2. Time to reach four stages of recovery (first breath, first attempt to right, completion of righting reflex and crawl) graphed as percentages of time required by littermate controls. Results of single experiments indicated by positions of short horizontal lines on thin verticle lines. Means of ATP animals connected by broken line. As shown by lines indicating three standard errors of the means (3 SE), the results are highly significant. Data from Miller, Miller and Farrar, 1950.

TABLE 8. EFFECTS OF COMBINING HYPOTHERMIA, NEMBUTAL AND HYDROGEN PEROXIDE

	PERCENT OF CONTROL T.O.D.	PERCENT OF H_2O CONTROL T.O.D.	PERCENT OF HYPOTHERMIC + PEROXIDE T.O.D.
Control—Normothermic	100%		
Control—Normothermic + H_2O	124%	100%	
Hypothermic + peroxide	329%	265%	100%
Hypothermic + nembutal + peroxide	543%	437%	162%

mates cooled to 20° and injected with peroxide had survival times increased to 329% of that of the uninjected control, 265% of that of the water-injected control. The fourth member of each litter was cooled and received Nembutal as well as peroxide. The mean survival times of these were 543% of the uninjected control, 437% of the water-injected control, and 162% of the animals who were cooled and given peroxide. The fifth mem-

ber of each litter was cooled and given both Nembutal and peroxide. It was removed from the chamber at the time of death of the cooled, peroxide-injected littermate. In every case it recovered completely from 265% of the lethal exposure for the uninjected normothermic control.

SUMMARY

To recapitulate, evidence from studies with deep hypothermia shows that tissues and even large pieces of organs if properly dehydrated with glycerol can be cooled to −79° or lower without severe damage. Tissue culture studies show that temperatures in the range from +5° to 0° are best for preserving embryonic and adult organs without the use of glycerol. Recent studies with adult organisms show that under proper conditions they will tolerate 0° for a matter of hours without apparent damage and with complete recovery of function upon rewarming. The principal danger at present is ventricular fibrillation in the range between about 25° and 13° when cardiac arrest appears. Recent studies indicate that we may expect an early solution to this problem. When this happens wide fields of application for hypothermia will be open for exploration.

The application of hypothermia to the treatment of asphyxia both in the adult and newborn has a solid basis of experimental results. These have shown that not only does cooling prolong asphyxial survival, it can prevent death from asphyxia. In addition, hypothermia potentiates the beneficial effects of narcosis, hydrogen peroxide and combinations of these.

REFERENCES

1. Adolph, E. F. Tolerance to cold and anoxia in infant rats. *Am. J. Physiol.* 155: 366–377, 1948.
2. Adolph, E. F. Responses to hypothermia in several species of infant mammals. *Am. J. Physiol.* 166: 75–91, 1951.
3. Albaum, H. G. and H. I. Chinn. Brain metabolism during acclimatization to high altitude. *Am. J. Physiol.* 174: 141–145, 1953.
4. Alexander, L. The Treatment of Shock From Prolonged Exposure to Cold, Especially in Water. Office of Publ. Board, Dept. of Commerce, Washington, D. C. Report No. 250, 1945.
5. Allen, F. M. Local asphyxia and temperature changes in relation to gangrene and other surgical problems. *Tr. A. Am. Physicians* 52: 189–194, 1937.
6. Allen, F. M. The tourniquet and local asphyxia. *Am. J. Surg.* 41: 192–200, 1938.
7. Allen, F. M. Resistance of peripheral tissues to asphyxia at various temperatures. *Surg. Gynec. & Obstet.* 67: 746–751, 1938.
8. Allen, F. M. Ligation and refrigeration of intestine. *Surgery* 3: 893–898, 1938.
9. Allen, F. M. Effects of ligation on nerves of the extremities. *Ann. Surg.* 108: 1088–1093, 1938.
10. Allen, F. M. Physical and toxic factors in shock. *Arch. Surg.* 38: 155–180, 1939.
11. Allen, F. M. Surgical considerations of temperature in ligated limbs. *Am. J. Surg.* 45: 459–464, 1939.

12. Allen, F. M. Refrigeration anesthesia for limb operations. *Anesthesiology* 4: 12–16, 1943.
13. Allen, F. M. Experiments on theory and therapy of shock. *Arch. phys. Therap.* 24: 327–335, 1943.
14. Allen, F. M. Treatment of surgical shock and embolism. *J. Internat. Coll. Surgeons* 7: 423–434, 1944.
15. Allen, F. M. Broader aspects of refrigeration anesthesia. *Anesth. & Analg.* 24: 51–65, 1945.
16. Allen, F. M. Refrigeration in general surgery of limbs. *Am. J. Surg.* 68: 170–184, 1945.
17. Allen, F. M. Refrigeration in obstetric crises. *West J. Surg.* 56: 548–551, 1948.
18. Allen, F. M. Hypothermic anesthesia. *Anesth. & Analg.* 29: 97–100, 1950.
19. Allgöwer, M. and T. G. Blocker, Jr. Viability of skin in relation to various methods of storage. *Texas Rep. Biol. & Med.* 10: 3–21, 1954.
20. Andjus, R. K. Suspended animation in cooled, supercooled and frozen rats. *J. Physiol.* 128: 547–556, 1955.
21. Andjus, R. K., and J. E. Lovelock. Reanimation of rats from body temperatures between 0° and 1°C by microwave diathermy. *J. Physiol.* 128: 541–546, 1955. between 0°C by microwave diathermy. *J. Physiol.* 128: 541–546, 1955.
22. Andjus, R. K., and A. U. Smith. Reanimation of adult rats from body temperatures between 0° and 2°C. *J. Physiol.* 128: 446–472, 1955.
23. Antoschkina, E. D. Uber die Ausbildung der Wärmeregulierung im Laute der Ontogenese. *Fiziol. Zhur. U.S.S.R.* 26: 3–15, 1939.
24. Arnott, J. *On the Remedial Agency of a Local Anesthetic or Benumbing Temperature in Various Painful and Inflammatory Diseases.* London: Churchill, 1851, 54 pp.
25. Badeer, H. Influence of oxygen on hypothermic cardiac standstill in the heart-lung preparation. *Circulation Res.* 3: 28–31, 1955.
26. Bailey, C. J. Present status of the problem. Chapter 1. In: *Neurological and Psychological Deficits of Asphyxia Neonatorum.* W. F. Windle, Ed. Springfield: Thomas 1957.
27. Bailey, C. P., B. A. Cookson, D. F. Downing and W. B. Neptune. Cardiac surgery under hypothermia. *J. Thoracic Surg.* 27: 73–91, 1954.
28. Bajenova, J. A. Culture des Tissus de la Cornée Conservée. *Arch. d'Ophtal.* 53: 300–307, 1936.
29. Beattie, E. J. Jr., D. Adovasio, J. M. Keshishian and B. Blades. Refrigeration in experimental surgery of the aorta. *Surg. Gynec. & Obst.* 96: 711–713, 1953.
30. Becker, R. F. Some observations on learning ability in the guinea pig. *Quart. Bull. Northwestern Univ. M. School.* 20: 318–328, 1946.
31. Becker, R. F., and W. Donnell. Learning behavior in guinea pigs subjected to asphyxia at birth. *J. Comp. & Physiol. Psychol.* 45: 153–162, 1952.
32. Bigelow, W. G., W. K. Lindsay and W. F. Greenwood. Hypothermia, its possible role in cardiac surgery: An investigation of factors governing survival in dogs at low body temperatures. *Ann. Surg.* 132: 849–866, 1950.
33. Bigelow, W. G., W. K. Lindsay, R. C. Harrison, R. A. Gordon and W. F. Greenwood. Oxygen transport and utilization in dogs at low body temperatures. *Am. J. Physiol.* 160: 125–137, 1950.
34. Billingham, R. E. The Storage of Skin. In: *Preservation and Transplantation of Normal Tissues.* Boston: Little, Brown 1954, pp. 160–171.
35. Blundell, W. *Painless Tooth Extraction Without Chloroform; With Observa-*

tions on Local Anaesthesia by Congelation in General Surgery. London: Churchill, 1854, 64 pp.

36. Boerema, I., A. Wildschut, W. J. H. Schmidt and L. Broekhuysen. Experimental researches into hypothermia as aid in surgery of heart. *Arch. chir. neerl.* 3: 25–34, 1951.
37. Bright, J. W. *Cancer, its Classification and Remedies*. Philadelphia: Butter, 1871.
38. Britton, S. W. Effects of lowering the temperature of homeothermic animals. *Quart. J. Exper. Physiol.* 13: 55, 1922.
39. Brokaw, R., and K. E. Penrod. Bromsulphalein removal rates during hypothermia in the dog. *Am. J. Physiol.* 159: 365–368, 1949.
40. Bucciante, L. Sopravvivenza dei tessuti embrionali di pollo mantenuti in liquido di Ringer a bassa temperature. *Atti. R. Accad. Naz. Lincei Rend. Cl. Sci. Fis. Mat. e Nat.* 14: 356–361, 1931 (*Biol. Abstr.* 8: #12184).
41. Callahan, J. C., D. A. McQueen, J. W. Scott and W. G. Bigelow. Cerebral effects of experimental hypothermia. *Arch. Surg.* 68: 208–215, 1954.
42. Carrel, A. The preservation of tissues and its application in surgery. *J.A.M.A.* 59: 523–526, 1912.
43. Chang, M. C. Resumption of heartbeat in the rabbit embryo after being kept at 10° or 0° for several days. *Fed. Proc.* 13: 25, 1954.
44. Cooke, T. W. *On Cancer, Its Allies and Counterfeits*. London: Longmans, Green 1865.
45. Cookson, B. A., W. Neptune and C. P. Bailey. Intracardiac surgery with hypothermia. *J. Internat. Coll. Surgeons* 18: 685–694, 1952.
46. Cookson, B. A., W. Neptune and C. P. Bailey. Hypothermia as a means of performing intracardiac surgery under direct vision. *Dis. Chest* 22: 245–260, 1952.
47. Crossman, L. W., and F. M. Allen. Principles of surgical and therapeutic refrigeration. *S. Clin. North America* 25: 361–370, 1945.
48. Crossman, L. W., and F. M. Allen. Shock and refrigeration. *J.A.M.A.* 130: 185–189, 1946.
49. Crossman, L. W. Refrigeration uses in surgery. *Med. Rec. & Ann.* 44: 131–138, 1950.
50. Crossman, L. W. The status of refrigeration for amputations and for tissue preservation. *Am. J. Surg.* 91: 92–98, 1956.
51. Currie, J. Medical Reports on the Effects of Water, Cold and Warm, as a Remedy in Fever and Other Diseases, Whether Applied to the Surface of the Body or Used Internally. London: Cadell, Davies 1805, ed. 4, vol. 1.
52. D'Amato, H. E., and A. H. Hegnauer. Blood volume in the hypothermic dog. *Am. J. Physiol.* 173: 100–102, 1953.
53. Deansely, R. Immature rat ovaries grafted after freezing and thawing. *J. Endocrinol.* 11: 197–200, 1954.
54. Deansley, R. Spermatogenesis and endocrine activity in grafts of frozen and thawed rat testis. *J. Endocrinol.* 11: 201–206, 1954.
55. Deansley, R. Histological evolution of rat gonadial tissue transplanted after freezing and thawing. In: *Preservation and Transplantation of Normal Tissues*. Boston: Little, Brown 1954, pp. 86–93.
56. Delorme, E. J. Experimental cooling of the blood stream, *Lancet II*, 914–915, 1952.
57. Domini, G. *Atti regia accad. fisiocritici Siena* 2: 443, 1927. (quoted by Hiestand *et al.*, 1952).
58. Downing, D. F., B. A. Cookson, K. K. Keown and C. P. Bailey. Hypothermia in cardiac surgery. *J. Pediat.* 44: 134–144, 1954.

59. Dundee, J. W., W. E. B. Scott and P. R. Mesham. The production of hypothermia. *Brit. Med. J.* II: 1244–1246, 1953.
60. Edwards, W. F. *De l'influence des agents physiques sur la vie.* Paris: Crochard, 1824, 630 pp.
61. Edwards, W. S., S. Tulay, W. E. Reber, A. Siegal and R. J. Bing. Coronary blood flow and myocardial metabolism in hypothermia. *Ann. Surg.* 139: 275–281, 1954.
62. Fairfield, J. Effects of cold on infant rats: body temperatures, oxygen consumption, electrocardiograms. *Am. J. Physiol.* 155: 355–365, 1948.
63. Falkmer, S., and K. Kjellgren. Morphologic and functional changes under hypothermia. An experimental study on rabbits with special reference to the liver. *Acta. Soc. med. upsalien.* 60: 199–223, 1955.
64. Fay, T. Observations on prolonged human refrigeration. *New York J. Med.* 40: 1351–1354, 1940.
65. Fay, T., and G. C. Henny. Correlation of body segmental temperatures and its relations to the location of carcinomatous metastasis. *Surg. Gynec. & Obstet.* 66: 512–524, 1938.
66. Fazekas, J. F., F. A. D. Alexander and H. E. Himwich. Tolerance of the newborn to anoxia. *Am. J. Physiol.* 134: 281–287, 1941.
67. Fazekas, J. F., and H. E. Himwich. Effect of hypothermia on cerebral metabolism. *Proc. Soc. Exper. Biol. & Med.* 42: 537–538, 1939.
68. Fleming, R. Acid-base balance of the blood in dogs at reduced body temperature. *Arch. Surg.* 68: 145–152, 1954.
69. Frank, O. Einfluss der Herztemperatur auf die erregbarkeit der beschleunigenden und verlangsamenden Nerven. *Ztschr. Biol.* 49: 392–420, 1907.
70. Geiger, J. General crymotherapy: a symposium. Basal metabolism determinations. *Bull. New York Acad. Med.* 16: 323–324, 1940.
71. Glass, H. G., F. F. Snyder and E. Webster. The rate of decline in resistance to anoxia of rabbits, dogs, and guinea pigs from the onset of viability to adult life. *Am. J. Physiol.* 140: 609–615, 1944.
72. Gollan, F. Cardiac arrest of one hour duration in dogs during hypothermia of 0°C followed by survival. *Fed. Proc.* 13: 57, 1954.
73. Gollan, F., P. Blos and H. Schuman. Exclusion of heart and lungs from circulation in the hypothermic closed-chest dog by means of a pump-oxygenator. *J. Appl. Physiol.* 5: 180–190, 1952.
74. Gollan, F., E. C. Hamilton and G. R. Meneely. Consecutive survival of open chest hypothermic dogs after prolonged by-pass of heart and lungs by means of a pump-oxygenator. *Surgery* 35: 88–97, 1954.
75. Gollan, F., J. E. Hoffman and R. M. Jones. Maintenance of life of dogs below 10°C without hemoglobin. *Am. J. Physiol.* 179: 640, 1954.
76. Gollan, F., J. T. Grace, M. W. Schell, D. S. Tysinger and L. B. Feaster. Left heart surgery in dogs during respiratory and cardiac arrest at body temperatures below 10°C. *Surgery* 38: 363–372, 1955.
77. Gollan, F., R. Phillips, J. T. Grace and R. M. Jones. Open left heart surgery in dogs during hypothermic asystole with and without extracorporeal circulation. *J. Thoracic. Surg.* 30: 626–630, 1955.
78. Gollan, F., D. S. Tysinger Jr., J. T. Grace, R. C. Kory and G. R. Meneely. Hypothermia of 1.5°C in dogs followed by survival. *Am. J. Physiol.* 181: 297–303, 1955.
79. Gosselin, R. E. Acute hypothermia in guinea pigs. *Am. J. Physiol.* 157: 103–115, 1949.

80. Gray, S. W. Rat survival time during drowning after immersion. *J. Appl. Physiol.* 8: 245–248, 1955.
81. Green, H. N., and H. B. Stoner. Biological actions of the adenine nucleotides. London: Lewis, 1950.
82. Gruner, O. C. A treatise on the canon of medicine of Avicenna, incorporating a translation of the first book. London, Paragraph 1075, 1930.
83. Harreveld, A. van, and D. B. Tyler. Metabolism of asphyxiated spinal cord. *Am. J. Physiol.* 138: 140–148, 1942.
84. Hegnauer, A. H., and K. E. Penrod. Observations on the pathologic-physiology in the hypothermic dog. *USAF. Tech. Rept. No. 5912.* (Wright-Patterson Air Force Base, Dayton, Ohio) 1950, 108 pp.
85. Hegnauer, A. H., J. Flynn and H. D'Amato. Cardiac physiology in dog during rewarming from deep hypothermia. *Am. J. Physiol.* 107: 69–75, 1951.
86. Hegnauer, A. H., and H. D'Amato. Oxygen consumption and cardiac output in the hypothermic dog. *Am. J. Physiol.* 178: 138–142, 1954.
87. Hegnauer, A. H., W. J. Schriber and H. O. Haterius. Cardiovascular response of the dog to immersion hypothermia. *Am. J. Physiol.* 161: 455–465, 1950.
88. Hetherington, D. C., and J. S. Graig. Tolerance of chick heart tissues to a time-temperature factor before explantation in tissue culture. *J. Cell. & Comp. Physiol.* 14: 197–203, 1939.
89. Hicks, C. E., M. C. McCord and S. G. Blount, Jr. Electrocardiographic changes during hypothermia. *Circulation* 13: 21–28, 1956.
90. Hiestand, W. A., F. W. Stemler and J. E. Wiebers. Effect of ambient water temperature, alcohol, and certain endocrine products on survival from drowning in rats and mice. *Am. J. Physiol.* 169: 108–110, 1952.
91. Himwich, H. E., A. O. Bernstein, H. Herrlich, A. Chesler and J. F. Fazekas. Mechanisms for the maintenance of life in the newborn during anoxia. *Am. J. Physiol.* 135: 387–391, 1942.
92. Hufnagel, C. A. Experimental and clinical observations on the transplantation of blood vessels. In: *Preservation and Transplantation of Normal Tissues.* Boston: Little Brown, 1954, pp. 196–206.
93. Hufnagel, C. A., and H. H. G. Eastcott. The preservation of arterial grafts by freezing. *Lancet* I: 531–537, 1952.
94. Hurder, W. P. Changes in maze performance in rats following exposure to anoxia. *J. Comp. & Physiol. Psychol.* 44: 473–478, 1951.
95. Hurder, W. P. Quantitative cortical changes produced by anoxia in rats. *J. Comp. & Physiol. Psychol.* 45: 362–367, 1952.
96. Hurder, W. P., and A. F. Sanders. The effects of neonatal anoxia on the maze performance of adult rats. *J. Comp. & Physiol. Psychol.* 46: 61–63, 1953.
97. Jackson, F. K., and A. Alonge. What constitutes a lethal reduction of temperature? *Am. J. Physiol.* 109: 447–449, 1934.
98. Juvenelle, A. A., J. Lind and C. Wegelius. Quelques possibilités offertes par l'hypothermie générale profonde provoquée. *La Presse Medicale*, no. 44: 973–978, 1952.
99. Juvenelle, A. A., J. Lind and C. Wegelius. A new method of extracorporeal circulation. Deep hypothermia combined with artificial circulation. *Am. Heart J.* 47: 692–736, 1954.
100. Knocker, P. Effects of experimental hypothermia on vital organs. *Lancet* II: 837–840, 1955.
101. Laborit, H. L'hibernation artificielle dans le traitement des états de choc. Marseille Chir. 3: 1–15, 1953.

102. Laborit, H., and P. Huguenard. Les ganglionplegiques et l'hibernation artificielle en chirurgie. *Journées Therapeut. Paris.* 1953. pp. 115–133.
103. Laufman, H. Profound accidental hypothermia. *J.A.M.A.* 147: 1201–1212, 1951.
104. Lewis, F. J., and M. Taufic. Closure of atrial septal defects with the aid of hypothermia. Experimental accomplishments and report of one successful case. *Surgery* 33: 52–59. 1953.
105. Lewis, R. B., and R. M. Thompson. Nephrosis following experimental local cold injury. *Milit. Surg.* 109: 518–530, 1951.
106. Lewis, T. Observations on some normal and injurious effects of cold upon skin and underlying tissues; etc. *Brit. Med. J.* 2: 795–797, 837–839, 869–871, 1941.
107. Lovelock, J. E. Resuspension in plasma of human red blood-cells frozen in glycerol. *Lancet* I: 1238–1239, 1952.
108. Lovelock, J. E. The haemolysis of human red blood-cells by freezing and thawing. *Biochim. et biophys. acta* 10: 414–426, 1953.
109. Lovelock, J. E. The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. *Biochim. et biophys. acta* 11: 28–36, 1953.
110. Lovelock, J. E. Biophysical aspects of the freezing and thawing of living cells. *Proc. Roy. Soc. Med.* 47: 60, 1954.
111. Marshall, S., J. C. Owens and H. Swan. Temporary circulatory occlusion to the brain of the hypothermic dog. *Arch. Surg.* 72: 98–106, 1956.
112. Meidinger, O. Versuche über den Tod des Erfrierens. *Ztschr. Biol.* 100: 361–372, 1940.
113. Meneely, G. R., S. E. Stephenson, Jr. and O. M. Kochitsky. The efficacy of quinidine in preventing ventricular fibrillation induced by temporary coronary occlusion in dogs. *Am. J. Med.* 9: 403, 1950.
114. Miller, F. S., and J. A. Miller, Jr. Effects of hypothermia on neonatal asphyxia and its sequellae. *Bull. Ga. Acad. Sci.* 11: 54–56, 1953.
115. Miller, J. A., Jr. Studies on heteroplastic transplantation in triclads. *Anat. Rec.* 67: 56, 1936.
116. Miller, J. A., Jr. Studies on heteroplastic transplantation in triclads. I. Cephalic grafts between *Euplanaria dorotocephala* and *E. tigrina*. *Physiol. Zool.* XI: 214–247, 1938.
117. Miller, J. A., Jr. Factors in neonatal resistance to anoxia. I. Temperature and survival of newborn guinea pigs under anoxia. *Science* 110: 113–114, 1949.
118. Miller, J. A., Jr. Cold as a means of combatting asphyxia in newborn guinea pigs. *Col. Net* 19: 17–19, 1949.
119. Miller, J. A., Jr. Effects of variations in body temperature upon resistance to asphyxia in the neonatal guinea pig. *Cold Spring Harbor Symp. on Quant. Biol.* 19: 152–155, 1954.
120. Miller, J. A., Jr. Factors influencing survival after neonatal asphyxia. Chapter 7. In: *Neurological and Psychological Deficits of Asphyxia Neonatorum.* W. F. Windle, Ed. Springfield: Thomas 1957.
121. Miller, J. A., Jr., and F. S. Miller. The effects of narcosis combined with hypothermia on the resistance of neonatal guinea pigs to asphyxia. *Anat. Rec.* 117: 588, 1953.
122. Miller, J. A., Jr., and F. S. Miller. Factors in neonatal resistance to anoxia. II. Effects of elevated and reduced temperature upon survival and recovery by neonatal guinea pigs. *Surgery* 36: 916–931, 1954.
123. Miller, J. A., Jr., and F. S. Miller. Effects of narcosis and hypothermia upon resistance to asphyxia in newborn guinea pigs. (A Kodachrome moving picture.) *Anat. Rec.* 124: 478–479, 1956.

124. Miller, J. A., Jr., and F. S. Miller. Factors in neonatal resistance to anoxia. III: The potentiation by narcosis of the effects of hypothermia in the neonatal guinea pig. In preparation.
125. Miller, J. A., Jr., F. S. Miller and C. B. Farrar. Hydrogen peroxide as a source of oxygen for asphyxiated newborn guinea pigs. *Anat. Rec.* 105: 85, 1949.
126. Miller, J. A., Jr., F. S. Miller and C. B. Farrar. Some effects of adenosinetriphosphate upon survival and spontaneous recovery from asphyxia in newborn guinea pigs. *Anat. Rec.* 106: 58, 1950.
127. Miller, J. A., Jr., F. S. Miller and C. B. Farrar. Effects of temperature upon resistance of guinea pigs to anoxia. *Fed. Proc.* 10: 92, 1951.
128. Mollison, P. L., and H. A. Sloviter. Successful transfusion of previously frozen human red cells. *Lancet* II: 862–864, 1951.
129. Mollison, P. L., H. A. Sloviter and H. Chaplin, Jr. Survival of transfused red cells previously stored for long periods in the frozen state. *Lancet* II: 501–505, 1952.
130. Nedzel, A. J. Effects of body chilling upon the blood vessels of denervated and intact kidneys in dogs and rabbits. *J. Aviation Med.* 23: 49–53, 1952.
131. Nunn, T. W. Application of cold as an anaesthetic agent in operations for removing warty excrescences. *Lancet* II: 262, 1850.
132. Osborn, J. J. Experimental hypothermia. Respiratory and blood pH changes in relation to cardiac function. *Am. J. Physiol.* 175: 389–398, 1953.
133. Owens, J. C., A. E. Prevedel and H. Swan. Prolonged experimental occlusion of thoracic aorta during hypothermia. *Arch. Surg.* 70: 95–97, 1955.
134. Paltauf, R. M. General crymotherapy: a symposium. Biopsies and death. *Bull. New York Acad. Med.* 16: 332–334, 1940.
135. Parkes, A. S. Preservation of human spermatozoa at low temperatures. *Brit. Med. J.* II: 212–213, 1945.
136. Parkes, A. S. Storage of mammalian spermatozoa at low temperatures. *Proc. Soc. Study Fertility* 2: 12, 1951.
137. Parkes, A. S. Preservation of spermatozoa, red blood-cells and endocrine tissue at low temperatures. *Freezing and Drying* 1952, p. 99. (Publ. by Institute of Biol.)
138. Parkes, A. S., and A. U. Smith. Effects of low temperatures on ovarian tissue. *J. Endocrin.* 7: XXXIX, 1951.
139. Parkes, A. S., and A. U. Smith. Regeneration of rat ovarian tissue grafted after exposure to low temperatures. *Proc. Roy. Soc., London, S. B,* 140: 455, 1953.
140. Parkes, A. S., and A. U. Smith. Storage of testicular tissue at very low temperatures. *Brit. Med. J.* I: 315–316, 1954.
141. Pembrey, M. S., and W. H. White. The regulation of temperature in hybernating animals. *J. Physiol.* 19: 477–495, 1896.
142. Penrod, K. E. Oxygen consumption and cooling rate in immersion hypothermia in the dog. *Am. J. Physiol.* 157: 436–444, 1949.
143. Pepper, F. J. Studies on viability of mammalian skin autografts after storage at different temperatures. *Brit. J. Plastic Surg.* 6: 250–256, 1954.
144. Perrin, M., and L. Lallemand. *Traité d'anesthésie chirurgicale.* Paris: Chamerot, 1863.
145. Pierce, E. C. II, R. E. Gross, A. H. Bill, Jr. and J. K. Merrill, Jr. Tissue culture evaluation of the viability of blood vessels stored by refrigeration. *Ann. Surg.* 129: 333–348, 1949.
146. Pillsbury, S. 14,501 deliveries with no maternal death. *Calif. Med.* 79: 343–345, 1953.

147. Polge, C. Functional survival of fowl spermatozoa after freezing at −79°C. *Nature, London* 167: 949–950, 1951.
148. Polge, C. The preservation of spermatozoa at low temperatures. In: *Mammalian Germ Cells*, Ciba Foundation Symposium. 1953, pp. 108–118.
149. Polge, C., and A. S. Parkes. Possibilities of long-term storage of spermatozoa at low temperatures. *Anim. Breeding Abstr.* 20: 1, 1952.
150. Polge, C., and L. E. A. Rowson. Fertilizing capacity of bull spermatozoa after freezing at −79°C. *Nature, London* 169: 626, 1952.
151. Polge, C., A. U. Smith and A. S. Parkes. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature, London* 164: 1136, 1949.
152. Pomerat, C. M., and R. B. Lewis. Thermal stress and tissue culture. *Texas Rep. Biol. & Med.* 11: 307–346, 1953.
153. Prevedel, A. E., V. Montgomery and H. Swan. Effect of coronary perfusion of prostigmine on ventricular fibrillation in the hypothermic dog. *Proc. Soc. Exper. Biol. & Med.* 85: 596–597, 1954.
154. Ramos, J. G. Ventricular fibrillation in artificial hypothermia. *J. Thoracic Surg.* 31: 635–642, 1956.
155. Reissman, K. R., and S. Kapoor. Dynamics of hypothermic heart muscle (heart lung preparation). *Am. J. Physiol.* 184: 162–170, 1956.
156. Robb, C., and H. H. G. Eastcott. The preservation of arteries and other tissues for clinical use. In: *Preservation and Transplantation of Normal Tissues*. Boston: Little, Brown 1954, pp. 190–195.
157. Rosmoff, H. L., and D. A. Holaday. Cerebral blood flow, and cerebral oxygen consumption during hypothermia. *Am. J. Physiol.* 179: 85–88, 1954.
158. Ross, D. N. Hypothermia. *Guy's Hosp. Rep.* 103: 97–138, 1954.
159. Rowson, L. E. A., and C. Polge. Storage of bull semen at −79°C and fertility results for up to 12 months. *Vet. Rec.* 65: 677, 1953.
160. Rycroft, B. W. The present state of corneal grafts. In: *Preservation and Transplantation of Normal Tissues*. Boston: Little, Brown, 1954, pp. 210–218.
161. Safford, F. K., and M. B. Nathanson. Clinical observations on tissue temperatures. *Arch. Surg.* 49: 12–22, 1944.
162. Severinus, M. A. De efficaci medicina libri iii. Frankfurt: 1646. (Quoted from Fulton, J. F. *The Centennial of Surgical Anesthesia: An Annotated Catalogue of Books and Pamphlets Bearing on the Early History of Surgical Anesthesia*. New York: Henry Schuman, 1946, XV, 102 pp.)
163. Simpson, S., and P. T. Herring. Effect of cold narcosis on reflex action in warm blooded animals. *J. Physiol.* 32: 305–311, 1905.
164. Sircar, P. Plasma volume, bleeding and clotting time on hypothermic dogs. *Proc. Soc. Exp. Biol. & Med.* 87: 194–195, 1954.
165. Sloviter, H. A. Recovery of human red blood cells after freezing. *Lancet* I: 823–824, 1951.
166. Sloviter, H. A. In vivo survival of rabbit's red cells recovered after freezing. *Lancet* I: 1350, 1951.
167. Sloviter, H. A. Recovery of human red cells after prolonged storage at −79°C. *Nature, London* 169: 1013, 1952.
168. Smith, A. U. Prevention of haemolysis during freezing and thawing of red blood cells. *Lancet* II: 910–911, 1950.
169. Smith, A. U. Cultivation of ovarian granulosa cells after cooling to very low temperatures. *Exper. Cell. Res.* 3: 574, 1952.
170. Smith, A. U. Effects of low temperature on living cells and tissues. In: *Biological Applications of Freezing and Drying*. New York: Acad. Press, 1954.

171. Smith, A. U., and A. S. Parkes. Storage and homografting of endocrine tissues. In: *Preservation and Transplantation of Normal Tissues*. Boston: Little, Brown. 1954, pp. 76–85.
172. Smith, A. U., and C. Polge. Survival of spermatozoa at low temperatures. *Nature*, London 166: 668–669, 1950.
173. Smith, L. W. Pathologic changes observed in human tissues subjected to subcritical temperatures. *Arch. Path.* 30: 424–439, 1940.
174. Stone, L. S. Return of vision in transplanted adult salamander eyes after several days of refrigeration. *Proc. Soc. Exp. Biol. & Med.* 54: 44–45, 1943.
175. Swan, H., I. Zeavin, S. G. Blount, Jr. and R. W. Virtue. Surgery by direct vision in the open heart during hypothermia. *J.A.M.A.* 153: 1081–1085, 1953.
176. Swan, H., I. Zeavin, J. H. Holmes and V. Montgomery. Cessation of circulation in general hypothermia. I—Physiologic changes and their control. *Ann. Surg.* 138: 360–376, 1953.
177. Swan, H., R. W. Virtue, S. G. Blount and L. T. Kirchner, Jr. Hypothermia in surgery: analysis of 100 clinical cases. *Ann. Surg.* 142: 382–400, 1955.
178. Talbott, J. H. The physiologic and therapeutic effects of hypothermia. *New England J. Med.* 224: 281–288, 1941.
179. Talbott, J. H., W. V. Consolazio and L. J. Pecora. Hypothermia. *Arch. Int. Med.* 68: 1120–1132, 1941.
180. Tripp, H. D. The use of oxygen liberating substances in the treatment of anaerobic peritonitis in guinea pigs. *Am. J. Surg.* 71: 636–645, 1946.
181. Vaughn, A. M. Experimental hibernation of metastatic growths. *J.A.M.A.* 114: 2293–2298, 1940.
182. Virtue, R. W. *Hypothermic Anesthesia*. Springfield: Thomas, 1955, 62 pp.
183. Waterman, A. J. Viability of embryonic chick tissues following storage at low temperatures. *Growth* 8: 175–203, 1944.
184. Wechsler, R. L., R. D. Dripps and S. S. Kety. Blood flow and oxygen consumption of the human brain during anesthesia produced by thiopental. *Anesthesiology* 12: 308–314, 1951.
185. Windle, W. F. Asphyxia Neonatorum. Springfield: Thomas, 1950, 61 pp.
186. Windle, W. F., R. F. Becker and A. Weil. Alterations in brain structure after asphyxiation at birth—an experimental study in the guinea pig. *J. Neuropath. & Exper. Neurol.* 3: 224–238, 1944.
187. Woodruff, L. M. Survival of hypothermia by the dog. *Anesthesiology* 2: 410–420, 1941.

EFFECT OF LOW BODY TEMPERATURES ON RESPIRATORY GAS EXCHANGE IN THE ANESTHETIZED DOG

ARTHUR B. OTIS, *Department of Physiology, University of Florida, College of Medicine, Gainesville, Florida*

DURING THE PAST FEW YEARS a considerable amount of information has accumulated regarding the effect on various physiological processes of lowering the body temperature of the anesthetized dog. Much of the work involved has been carried out or given impetus by surgeons interested primarily in the application of hypothermia to clinical surgery. In this they have met with considerable success, and at the same time they have contributed information and revealed problems of interest to the general physiologist.

The author's experience in this particular field is limited to some experiments done during the past year in collaboration with members of the Department of Surgery at the Johns Hopkins University, Dr. James Jude in particular. The purpose here is not to present the results of this work in detail, but rather to summarize in a general way what is known about the effect of lowering the body temperature on physiological processes involved in respiratory gas exchanges, and to mention in passing some problems of general physiological interest which remain largely unsolved.

Cooling of the anesthetized dog may be accomplished by immersion in ice water, by wrapping the body in a special blanket through which a coolant is pumped or by leading the circulation from a large artery through a cooling coil and returning it to a vein. The rate of cooling depends, of course, on many factors but is usually of the order of 10°C per hour.

METABOLISM

When a dog, anesthetized deeply enough to prevent shivering, is progressively cooled from a body temperature of 36° to 38° his oxygen consumption diminishes by about 5% of its initial value for each degree drop in body temperature (1, 2). Thus at a body temperature of 25°, oxygen consumption is only about 30% of normal and at temperatures below 20°C it is so small as to be difficult to measure with any precision by ordinary methods. If the anesthesia is not deep enough to prevent shivering, then during the initial phases of cooling the oxygen consumption may increase by as much as five fold, until a body temperature of about 32°C

is reached. Below this temperature shivering is largely abolished by the cold itself and oxygen consumption falls as described above (see fig. 1).

This diminished oxygen consumption is paralleled by a decreased carbon dioxide production, but our measurements were not sufficiently exact and well controlled to enable one to say definitely whether the metabolic respiratory quotient changes or remains constant as the temperature is lowered.

Such measurements would be of interest because they might give a clue as to whether cooling alters the metabolism qualitatively as well as quantitatively.

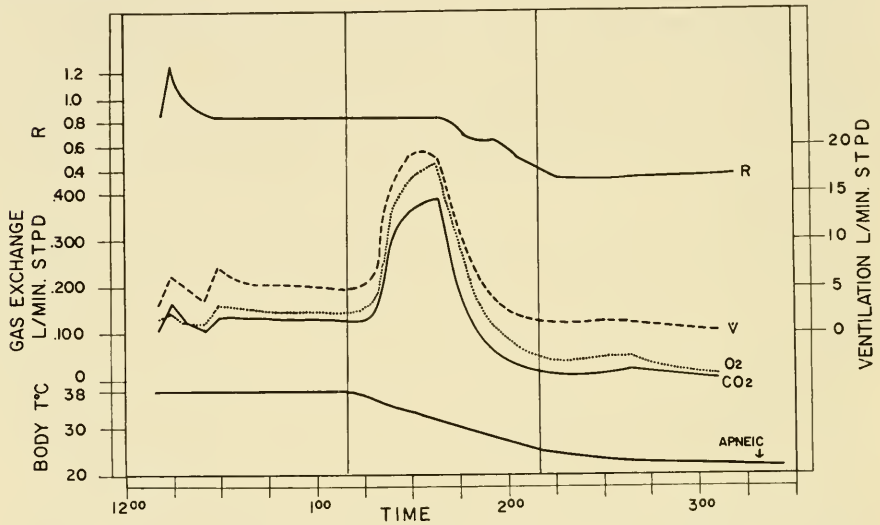


FIG. 1. Effect of cooling on pulmonary ventilation and gas exchange in a lightly anesthetized dog.

VENTILATION

The partial pressures of carbon dioxide and oxygen in the alveolar gas are determined by the ratios of alveolar ventilation to carbon dioxide elimination and to oxygen uptake respectively. During cooling, therefore, the partial pressures of these gases will remain unaltered if ventilation diminishes proportionately at the same rate as does metabolism. In the anesthetized dog, however, ventilation decreases during cooling relatively more than does metabolism, with hypoxia and respiratory acidosis as consequences. At a body temperature of between 20° and 25°C breathing ceases entirely and death occurs if artificial ventilation is not instituted (see fig. 1). This cessation of breathing is probably the manifestation of a functional failure somewhere in the neural mechanism controlling breath-

ing, but whether the primary failure is one of excitation, of conduction or of synaptic transmission is not known.

If the ventilation of the lungs is maintained at an adequate value by artificial means then dogs can sometimes survive for several hours at body temperatures of 20°C or even lower and show no signs of permanent injury on being rewarmed (1, 3).

DIFFUSION BETWEEN LUNG AND BLOOD

In normal circumstances ventilation and perfusion of the lung are so evenly distributed and transfer of carbon dioxide and oxygen by diffusion is so adequate that the partial pressures of these gases in the systemic arterial blood are close to being in equilibrium with those in the alveoli.

If lowering of the body temperature were to interfere in some way with this transfer, then a large arterial-alveolar gradient might be set up and respiratory gas exchange could be inadequate even though ventilation of the lungs were maintained. It has been suggested that some such impairment does actually occur (4) but neither we (5) nor Severinghaus *et al.* (6, 7) have been able to find any evidence for this point of view.

In our investigation of this problem we first of all made simultaneous measurements of alveolar and arterial partial pressures of carbon dioxide, at body temperatures covering the range from 16° to 37°C. On the average the values determined for arterial P_{CO_2} were 3 mm Hg higher than those for the corresponding alveolar values. This apparent gradient, which may reflect in part at least a small systematic error in one or both sets of measurements, need not concern us here. What is of importance to the present problem is that there was no significant or consistant difference between the apparent gradients at different temperatures.

Even though we found no evidence that low body temperatures produced any significant barrier to the transfer of CO_2 across the lung it was conceivable that O_2 transfer might be impaired because of the relatively low solubility of the latter gas. In collaboration with Dr. Jude an attempt was made to measure the diffusing capacity of the lung in dogs at normal and reduced body temperatures, using a steady state carbon monoxide method (5).

The diffusing capacity of the lung for a given gas is defined as the rate of transfer of that gas divided by the difference between its partial pressures in the alveoli and in the blood flowing through the pulmonary capillaries. The determination of diffusing capacities involves several assumptions, and relatively small analytical errors can lead to large variations in the final value obtained. However our four experiments, each on a different dog, were consistent in showing a drop in diffusing capacity with lowering of the body temperature, the decrement per degree drop

being about 5% of the initial value obtained at a normal body temperature. Since the decrement in total body oxygen consumption is of a similar magnitude, it appears that although the diffusing capacity at low temperatures is considerably decreased, it is still adequate for existing needs.

If the lung were a physical system having constant geometry we should expect the diffusing capacity to decrease by only about 1.2% per degree drop in temperature over the range involved in our experiments. Our observed decrease being about four times larger suggests that some physiological factor is involved in addition to purely physical ones. We have suggested that a decrease in the size and/or number of functioning pulmonary capillaries with a consequent decrease in area available for diffusion may be responsible.

CIRCULATION

If pulmonary ventilation and diffusing capacity are adequate the hemoglobin in the systemic arterial blood will be nearly fully saturated. This should be especially true at low temperatures because the affinity of hemoglobin for oxygen is increased; the dissociation curve is shifted to the left (8). Furthermore the amount of oxygen that can be carried in physical solution is increased at low temperatures. There is therefore a more than ample content of oxygen in the blood as it enters the tissue capillaries. Whether the tissues actually receive an adequate supply of oxygen depends now on whether the flow of this oxygen-rich blood is fast enough, i.e. whether the ratio of blood flow to oxygen consumption is maintained at a sufficiently high value.

The experimental evidence indicates that during cooling the frequency of the heart beat decreases in an approximately linear fashion while the stroke volume remains nearly constant (see fig. 2). The net result is a drop in cardiac output that is just about in proportion to the drop in oxygen consumption. Thus the venous blood oxygen content and the arterial-venous oxygen difference remain approximately constant during cooling (9, 10).

At body temperatures below 25°C ventricular fibrillation is a fairly common occurrence. The heart seldom regains its normal rhythm spontaneously and death from circulatory failure results. There are numerous theories which attempt to explain why the hypothermic heart is so prone to fibrillate, but none of them seems to be convincingly proven.

As long as fibrillation can be avoided the over-all total body blood flow appears to be quantitatively adequate for the transport of oxygen and carbon dioxide at temperatures down to 20°C or below. The over-all blood flows to the brain (11) and to the heart (12–14) are likewise sufficient as

judged by the maintenance of a normal arterial-venous oxygen difference at body temperatures down to 20°C.

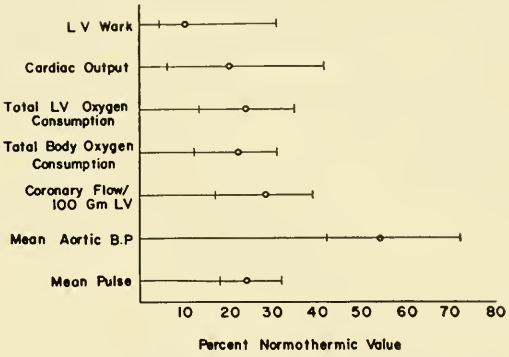


FIG. 2. Magnitude of various functions in the anesthetized dog at a body temperature of 20°C relative to those at normal body temperature. Circles indicate the means and the vertical lines the ranges of values obtained from measurements on 10 dogs.

AVAILABILITY OF OXYGEN TO TISSUES

Although it appears that the circulation carries plenty of oxygen to the tissues, the question remains as to whether the tissue cells are able to extract as much oxygen as they need from the capillary blood. The leftward shift of the dissociation curve that favors the loading of oxygen in the lungs also hinders the unloading in the tissues. If, as has been mentioned above, the percentage saturation of the venous blood with oxygen is similar at low and at normal temperatures, then the venous Po_2 must be lower at low temperatures because of the decreased dissociation. This means that there is either a diminution in the diffusion gradient for oxygen from capillary blood to tissue cells or in the Po_2 of the cells themselves. However, unless the reduction in diffusion gradient is proportionately no greater than the decrease in the requirement for oxygen by the cells, then ample oxygen will be supplied the cells and at a partial pressure similar to that which normally exists, provided that the diffusing capacity of the tissue is not diminished.

In some experiments by Hegnauer (10), oxygen was substituted for air as the inspired gas of hypothermic dogs. Although this must have increased the diffusion head for oxygen, no increase in oxygen consumption could be measured. Bigelow (1) kept dogs at 20°C for several hours and then rewarmed them, measuring oxygen consumption continuously. There was no evidence during the rewarming period that an oxygen debt was being repaid.

These measurements seem to show that the diffusion gradients in the tissues are on the whole adequate at temperatures as low as 20°C. However, they may not be sensitive enough to detect small changes and it is pos-

sible that in some localized and perhaps critical areas there is a deficient oxygen supply. This could come about, for example, if blood flow became too slow or stopped completely in local areas.

CO_2 TRANSPORT AND ACID BASE BALANCE

There are two principle effects of temperature on the carbonic acid-bicarbonate buffer system. A drop in temperature produces *1)* an increase in the solubility of CO_2 and *2)* a decrease in the apparent dissociation constant K′. If a sample of blood is collected and cooled under anaerobic conditions, it will become more alkaline, because the total concentration of CO_2 in its various forms cannot change and a greater proportion will exist in the undissociated form.

If, on the other hand, blood is kept in equilibrium with a constant P_{CO_2}, then the lower the temperature the higher will be the concentrations of both carbonic acid and bicarbonate in the blood. These changes occur in such a relationship to one another that at a constant P_{CO_2}, the pH of true plasma remains almost constant over a wide range of temperatures (15).

Physiologically, this means that if pulmonary ventilation is so controlled as to maintain a constant alveolar P_{CO_2} during cooling, the arterial pH will remain essentially constant unless a metabolic acidosis or alkalosis occurs. For an excellent discussion of this problem see Brewin *et al.* (16).

SUMMARY

Lowering the body temperature of the anesthetized dog produces a relatively greater reduction in ventilation than in oxygen consumption and CO_2 production, with an accompanying hypoxia and respiratory acidosis. At a temperature of about 20°–25°C breathing ceases entirely and asphyxial death results. If ventilation is continued by artificial means the circulation may continue to be adequate for respiratory gas transport at temperatures down to 15°C or lower, although ventricular fibrillation occurs rather frequently at temperatures below 25°C. Transfer of O_2 and CO_2 by diffusion probably remains adequate in both lung and tissues as long as ventilation and circulation are maintained, although there is a possibility that it may fail in localized regions of the body.

Figures 1 and 2 are based on data obtained in studies supported by funds provided under Contract AF41 (657)-30, with the School of Aviation Medicine, USAF, Randolph Field, Texas.

REFERENCES

1. Bigelow, W. G. et al. Oxygen transport and utilization in dogs at low body temperatures. *Am. J. Physiol.* 160: 125, 1950.

2. Sabiston, D., E. Theilen and D. Gregg. The relationship of coronary blood flow and cardiac output and other parameters in hypothermia. *Surgery* 38: 498, 1955.
3. Gollan, F. et al. Hypothermia of 1.5°C in dogs followed by survival. *Am. J. Physiol.* 181: 297, 1955.
4. Osbourne, J. J. Experimental hypothermia, respiratory and blood pH changes in relation to cardiac function. *Am. J. Physiol.* 175: 389, 1953.
5. Otis, A. and J. Jude. Effect of body temperature on pulmonary gas exchange. *Am. J. of Physiol.* In press.
6. Dripps, R. D. and J. W. Severinghaus. General anesthesia and respiration. *Physiol. Rev.* 35: 741, 1955.
7. Severinghaus, J. W. and M. Stupfel. Respiratory dead space increase following atropine in man, and atropine, vagal or ganglionic blockade and hypothermia in dogs. *J. Appl. Physiol.* 8: 81, 1955.
8. Brown, W. E. L. and A. V. Hill. The oxygen dissociation curve of blood and its thermodynamical basis. *Proc. Roy. Soc., London, S. B.* 94: 297, 1923.
9. Adolph, E. F. Effects of low body temperature on tissue oxygen utilization. Conference on the Physiology of Induced Hypothermia (sponsored by subcommittees on anesthesia, the cardiovascular system, shock and trauma of the committee on Medicine and Surgery), Washington, D. C., October 1955.
10. Hegnauer, A. Cold injury. Tr. 2nd conf. New York: Macy, 1952.
11. Rosomoff, H. L. and D. A. Holaday. Cerebral flow and cerebral oxygen consumption during hypothermia. *Am. J. Physiol.* 179: 85, 1954.
12. Berne, R. M. The effect of immersion hypothermia on coronary blood flow. *Circulation Res.* 2: 236, 1954.
13. Penrod, K. E. Cardiac oxygenation during severe hypothermia. *Am. J. Physiol.* 164: 79, 1951.
14. Jude, J. R., L. M. Haroutunian, and R. Folse. Hypothermic myocardial oxygenation. *Am. J. Physiol.* 190: 57, 1957.
15. Cullen, G. E., H. R. Keeler, and H. W. Robinson. The pK of the Henderson-Hasselbalch equation for hydrion concentration of serum. *J. Biol. Chem.* 66: 301, 1925.
16. Brewin, E. G., R. Gould, F. Nashat and E. Neil. An investigation of problems of acid-base equilibrium in hypothermia. *Guy's Hosp. Rep.* 104: 177, 1955.

Index